Evidence for a remote lung response in endotoxin-mediated direct lung injury: a strategic application of microarray technology.

By

Stephen Paul Smith BSc (Hons)

A thesis submitted for the degree of

Doctor of Philosophy

in the

College of Medicine and Veterinary Medicine

UNIVERSITY OF EDINBURGH

School of Medicine
University of Edinburgh

August, 2004

Declaration

DECLARATION

This thesis has been composed by myself and the work contained herein is my own

Stephen Paul Smith

Acknowledgments

I would like to thank, first and foremost, my primary supervisor Dr. David Collie. His patience, guidance, good cheer and constant willingness to spend time and effort has been what has made this project possible and I count myself as extraordinarily lucky to have worked with him and been supervised by him. I would also like to thank my co-supervisors Dr. Paul Dickinson and Dr. Varrie Ogilvie. Their expertise and knowledge have proved invaluable, particularly in the planning and application of the microarray analyses.

A number of people have been pivotal in the practical aspects of realising this project, and I would like to acknowledge the roles played by Alison Baker - who has more than lived up to her reputation as the oracle of all practical knowledge – along with Peter Tennant and Dr. Steve Tate for their assistance in the sample gathering process. I would also like to acknowledge the efforts of Richard Talbot and his team at ARK genomics for their expertise and hard work on the microarray and SSH experiments. Richard Blundell provided the pathologist's view of the sheep lung, for which I am very grateful.

This work was funded by the Medical Research Council who provided my stipend (and therefore prevented me from starving) and the Norman Salvesen Emphysema Research Trust who provided funding to cover research costs and consumables. Work was undertaken in the Wellcome Trust Centre for Research in Comparative Respiratory Medicine, whose director Professor Gordon Harkiss I would also like to thank.

None of this would have been possible without the support, help and occasional party my family and friends. Particular mention, of course, must be made of my parents and sister whose love, support and cheerful acceptance that I must be doing something clever has been much of the reason I am still (relatively) sane. Emotional support, cajoling, the reminder that there are much more difficult jobs in the world - but above all love happiness and good times were provided by Ellie, for which I will be eternally grateful.

Mention must also be made of my fellow PhD students, particularly Tom McNeilly who provided valuable advice on relaxation tactics and computer programming skills, and Ian Dacre who has been a shining example of organisation and industriousness in his approach to writing his own thesis. Thank you both for your friendship and good natured banter throughout the last three years. My thanks also to James, Tom C, Suzie and Tom W for helping pay my mortgage, as well as making life so much more pleasant with your company (yes, even James). Finally I would like to thank the Edinburgh University swimming and water polo club with whom I have spent a very considerable proportion of my free time over the last seven years, and who have provided me with a fund of memories, stories and a very big overdraft, you will all be missed (yes, even James).

Abstract

Endotoxin, a lipopolysaccharide component of the membrane of gram-negative bacteria, is responsible for rapid triggering of innate defence mechanisms in the lung. Such mechanisms, featuring local accumulation of neutrophil leucocytes, concomitant modulation of local immunoregulatory cytokine expression and the development of interstitial oedema are the hallmarks of an intense inflammatory response and comprise the clinical definition of acute lung injury (ALI). Although a proportion of individuals with ALI will progress towards a more severe form of lung injury referred to as the acute respiratory distress syndrome (ARDS) the mechanisms underlying such susceptibility are unknown. One potential avenue through which individuals may exhibit variable susceptibility to progression is in the nature and extent of the whole organ response to a local challenge. However, there is relatively little known in relation to whether the lung will mount such a 'remote' response to local challenge. The central hypothesis underlying this thesis is that the whole lung will respond to local challenge with endotoxin in the absence of any overt phenotypic change throughout the organ.

The work presented here is directed towards characterising the molecular aspects of this response using an ovine model. Quantitative RT-PCR analysis of the cytokine expression in directly challenged and contra lateral 'remote' lung segments showed that the lung produced the anti-inflammatory cytokine IL-10 remotely whilst the pro-inflammatory cytokines IL-1β, IL-6 and TNFα, which are associated with acute inflammation, were expressed in the directly challenged segment and correlated with the observed neutrophil influx to the challenged lung segment.

To further this characterisation a novel microarray platform was designed to specifically probe the genomic profile of the sheep lung. This process included the construction of a subtracted lung library and the identification of ovine homologs within a commercially available bovine genomic library. The selected elements were combined to make a cDNA microarray which was used to probe the nature of the remote response after LPS instillation. A remote response was observed.

The response was highly complex, involving mediators from diverse pathways and functional groups including cytoskeletal genes, ribosomal genes, mitochondrial products, prostaglandins and immune mediators. The characteristics of the transcriptional response in the remote segment were considered broadly anti-inflammatory in nature.

This study has shown for the first time that the lung responds as a whole organ to local inflammatory insult. Variation in the nature and extent of this remote response amongst individuals is proposed as a factor underlying susceptibility to progressive lung injury and ARDS. The study has also generated and utilised a novel ovine microarray, and produced a subtracted library of ovine lung sequences. Both of these represent considerable new resources for the study of pulmonary responses in both natural and experimental models of disease in sheep.

Table of Contents

List of figures

List of tables

Abbreviations

°C	Degree Celsius
A, C, G, T	Nucleotides: adenine, cytosine, guanine, thymine
ACS	American chemical society
ALI	Acute lung injury
Amp	Ampicillin
AMV	Avian myeloblastosis virus
ANF	Atrial neurotrophic factor
ARDS	Acute respiratory distress syndrome
aRNA	Amplified RNA
ATPase	Adenosine tri-phosphatase
BAC	Bacterial Artificial Chromosome
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BARC	Beltsville agricultural research center
BDNF	Brain derived neurotrophic factor
BLAST	Basic local alignment of sequences tool
bp	Base pair (s)
CD	Cluster determinant
cDNA	Complementary DNA
CNTF	Cerebral neurotrophic factor
CO_2	Carbon dioxide
COX	Cyclo-oxygenase
CPT	Carnitine palmitoyltransferase
$C_{(t)}$	Crossing threshold

Cy3/Cy5	Cyanine-3/cyanine-5
CYP	Cytochrome P
D. filaria	*Dictyocaulus filaria*
ddNTP	Dideoxynucleotide
DNA	Deoxynucleic acid
DNAse	Deoxynuclease
dNTP	Deoxynucleotide
ds DNA	Double stranded DNA
DTT	Dithiothreotol
EBVC	Easter Bush Veterinary Centre
E. coli	Escherichia coli
EDTA	Ethylenediamine Tetraacetic acid
e.g.	Exempli gratia (for example)
EGF	Epidermal growth factor
EMBL	European molecular biology Laboratory
EST	Expressed sequence tag
EtOH	Ethanol
Fig.	Figure
g	Acceleration due to gravity
h	Hour (s)
HCl	Hydrochloric acid
ICAM	Intra-cellular adhesion molecule
i.e.	Id est (that is)
IFN	Interferon
IGF	Insulin-like growth factor

IL	Interleukin
IPTG	Isopropyl-ß-d-thiogalactopyranoside
ISH	*In situ* hybridisation
Kan	Kanamycin
kb	Kilo base pairs
kDa	Kilo Dalton
kg	Kilogram
kPa	Kilo Pascal
LB	Luria-Bertoni broth
LBP	LPS binding protein
LiCl	Lithium chloride
LPS	Lipopolysaccharide
MARC	Meat animal research center
MCAO	Middle cerebral artery occlusion
M. capillaris	*Meullerius capillaris*
MHC	Major histo-compatibility complex
µg	Micrograms
µl	Microliter
µM	Micromolar
min	Minute (s)
ml	Milliliters
mM	Millimolar
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NCBI	National center for biotechnology information

NFκB	Nuclear factor κB
NGF	Neural growth factor
NH4Oac	Ammonium acetate
ng	Nanograms
nm	Nanometers
OD	Optical diameter
Oligo d (T)	Oligonucleotide deoxythymine
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PMN	Polymorphonuclear cell (neutrophil)
Pmol	Picomole
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Rotations per minute
RT	Reverse transcription
SDS	Sodium dodecyl sulfate
sec	Seconds
SP	Surfactant protein
ss DNA	Single stranded DNA
SSC	Standard saline citrate
SSH	Suppressive subtractive hybridisation
STAT	Signal transducer and activator of transcription
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA

TE	Tris-EDTA
TGF	Transforming growth factor
TIGR	Institute for genomic research
TIFF	Transferable image file format
Tm	Melting temperature
TNF	Tumour necrosis factor
Trk	Tyrosine kinase
U	Unit
Vol.	Volume

Chapter 1 General introduction

Rationale for thesis

Direct lung injury and the pathogenesis of the Acute Respiratory Distress Syndrome

The lung functions as the interface between air and blood, enabling oxygen uptake and carbon dioxide release for the process of respiration. As a consequence, the mucosal interface of the respiratory tract is exposed continuously to noxious agents, microbial organisms, particles, and allergens present in the environment and inhaled. An important evolved function of this interface is to prevent inhaled bacteria from causing infection. The first line of defence against such insult is provided by the innate immune response and is followed by the development of acquired immune responses associated with activation of T and B cells to specific antigens. In certain circumstances, such defences may be compromised and pneumonia (inflammation of the lung parenchyma) may develop. Such circumstances can include direct damage through pulmonary infection or aspiration, or may relate to sepsis (especially gram-negative), pancreatitis or multiple traumas.

The epithelium plays a central role in the immune and inflammatory response to lung challenge (Crestani & Aubier 1998) acting as the primary barrier between inhaled environmental factors, including pathogens and other inflammatory stimuli, and the circulation. Further, in addition to the barrier function, the bronchial and alveolar epithelia play a crucial role in mediating the acute inflammatory response to stimuli (Cooper et al. 2001).

Most people with pneumonia recover. However, in a proportion of susceptible individuals the condition progresses to diffuse pulmonary parenchymal injury

associated with non-cardiogenic pulmonary oedema and results in severe respiratory distress and hypoxemic respiratory failure. This progression is referred to as the acute respiratory distress syndrome (ARDS). Figures from a recent British Thoracic Society survey show that a total of 65,929 people died as a result of pneumonia in Great Britain in 1999 (British Thoracic Society 1999), making it the most common cause of death due to respiratory disease in that period. As highlighted by Pelosi et al (2003) the American-European Consensus Conference defined, in 1994, two pathogenetic pathways leading to ARDS: a direct ("primary" or "pulmonary") insult, that directly affects lung parenchyma, and an indirect ("secondary" or "extrapulmonary") insult, that results from an acute systemic inflammatory response (Pelosi et al. 2003). Distinct morphological aspects, respiratory mechanics and response to ventilatory strategies in patients with ARDS appear to reflect the pathogenetic pathway involved. Only a proportion of people with direct lung injury go on to develop ARDS and the exact proportions and levels of severity associated with progression remain highly variable and unpredictable. Understanding the mechanisms underlying this progression is key to identifying markers of susceptibility and potential prophylactic and/or therapeutic targets.

Large Animal Models of ARDS

Animal models have contributed significantly to our understanding of the pathophysiology of ARDS over the past 50 years (Brigham & Staub 1998). Early animal models of ARDS focussed on recreating the perceived clinical entity of 'non-cardiac oedema'. These models used chemicals to directly injure the lungs and increase vascular permeability leading to pulmonary oedema in the absence of raised vascular pressures. However, with the emerging consensus that the pathogenesis instead involved acute lung injury and inflammation, animal models began to focus

on inflammatory responses, particularly those following exposure to gram-negative bacteria and endotoxin.

Challenging hypotheses relating to the pathogenesis of non-cardiac oedema demanded the use of large animal models in which surgery and/or the placement of complex physiological instrumentation could be employed. Sheep figured prominently in this regard and the various modelling applications of this species have significantly contributed to the evolution of understanding away from oedema formation *per se* to inflammation as a consequence of exposure to gram negative bacteria and endotoxin. Indeed the first reports of altered vascular permeability as a consequence of *Pseudomonas aeruginosa* bacteraemia were reported some 30 years ago in this species (Brigham et al. 1974) and since this point in time there have been over 140 citations involving systemic endotoxin-induced lung injury in sheep, a reflection of the continued interest in this species as a relevant model for acute lung injury and ARDS.

In these models, systemic infusion of a single low dose of endotoxin causes pulmonary hypertension, hypoxemia, leucopoenia, reduced dynamic lung compliance and increased airway resistance. These changes are consistent with those seen in human sepsis and sepsis-induced extrapulmonary (or indirect) ARDS. The recent literature suggests that these models, whilst excellent models for the extrapulmonary ARDS phenotype, are of more limited value as models for the direct or pulmonary ARDS syndrome. This is a significant limitation as understanding the nature of progression from local direct lung injury to ARDS might offer therapeutic opportunities.

Direct lung injury, ARDS and the concept of whole organ susceptibility

Importantly, clues exist from other animal models that the consequence of direct lung insult may extend to change the susceptibility of the whole organ to further challenge. In particular *Pseudomonas* instillation in rats (Terashima et al. 1996) and acid instillation in a rabbit model (Miyazaki et al. 1999) have been used to model pulmonary ARDS and are reviewed below. Large animal models are of potential value in modelling whole organ responses to local challenge in that lung size and architecture favours discrimination of local and remote effects. Although the response of the lung to direct instillation of endotoxin has been studied in a number of large animal models, including sheep (Brogden, Ackermann, & Debey 1995), such models have largely failed to explore the mechanism of progression from local injury to ARDS.

With its placement at the air-blood interface it is accepted that the airway epithelium is highly susceptible to damage and will figure prominently in initiating any inflammatory response to local injury (Baeza-Squiban et al. 1999; Diamond, Legarda, & Ryan 2000; Holgate et al. 1999). Whilst the specific nature of the local response will vary according to the insult and the species involved, the principal characteristics of the response generally involve the activation of resident alveolar macrophages and neutrophils, local increases in inflammatory cytokines and chemokines in the bronchoalveolar space, and further activation and trafficking of neutrophils from the pulmonary circulation. However, the factors that both render an individual susceptible to progression and contribute to the maintenance of established ARDS are ill defined. It is perceived that with progressively more severe local inflammation the integrity of the alveolar-capillary barrier will be lost and local events will contribute, in the form of increased blood cytokine concentrations, to systemic inflammatory responses and

subsequent remote tissue damage. However, it is clear that there is a spectrum of susceptibility in individuals at risk for the development of ARDS and factors other than the severity of the local inflammatory event are likely to influence progression

In this regard, whilst the specific mechanisms remain undefined it is suggested that a change in the physiological 'state' of the whole organ facilitates the rapid progression, rather than a simple progressive spread from one or more initial foci of injury. Further, it is tempting to speculate that the nature and extent of any 'remote' response will vary amongst individuals and might indeed factor in the susceptibility of an individual's progress towards ARDS. Such a mechanism would seem intuitively reasonable in that, in addition to controlling the inflammatory process and implementing repair in the area subject to insult, there would be value in reducing the susceptibility to further insult in either adjacent or disparate parts of the system. However the converse, that a localised insult brings about a generalised increase in susceptibility is equally possible.

Indeed it is intriguing and significant that, in a rodent model of aspiration pneumonia, local lung instillation of acid significantly enhanced the inflammatory response of the contra lateral lung to LPS instillation at a later time point (24h). The implication is that an organ-wide response, in the form of increased susceptibility to LPS, was elicited by the initial local inflammatory event (Terashima et al. 1996).

Further, experimental gastric acid aspiration in rabbits rendered them more susceptible to develop an ARDS phenotype in response to increased ambient oxygen concentrations, suggesting an alteration of the ''set point'' for oxygen toxicity in the lungs, possibly by ''priming'' cells through activation of inflammatory pathways (Miyazaki et al. 1999).

Identifying the nature of such 'whole organ' responses to local lung challenge or indeed if they occur at all is the key objective of this thesis.

Lipopolysaccharide (LPS) and direct lung injury

Prior literature serves to indicate the validity of ovine models of ARDS based on systemic administration of lipopolysaccharide (LPS). With the stated objective of defining the whole organ response to local lung challenge and the lack of a validated model system to explore the mechanism of ARDS pathogenesis following direct lung insult it is appropriate to utilise this bacterial cell wall component as the basis of the insult. To this end a brief review of LPS in relation to known and anticipated effects in the lungs appears warranted.

Overview

Lipopolysaccharide (LPS) is a sugar-lipid component of the outer membrane of gram-negative bacteria (Redl et al. 1993) which is ubiquitous in the environment and to which the lung epithelium is continuously exposed (Vernooy et al. 2001). Gram-negative sepsis has been shown to be a primary cause of pneumonia (Standiford et al. 1996) and is implicated in the morbidity associated with ARDS (Terashima et al. 1996) and COPD exacerbation (Rennard 1999). Gram-negative bacteria are also involved in colonisation of the lungs of patients with cystic fibrosis and contribute significantly to tissue damage in this setting (De, V 2002). Experimental lung instillation of LPS has been used to model the above as well as effects analogous to toxic shock and environmentally initiated inflammation (Becker et al. 1999). LPS is recognised by receptors in the alveolar and bronchial epithelium (Levine 1995) as well as resident alveolar macrophages (Mantovani et al. 2001). These cells activate

the innate immune system in order to initiate and potentiate anti-bacterial responses as part of the acute-phase inflammatory response (Murtaugh et al. 1996).

Acute bacterial sepsis modelled by LPS instillation in the lung induces marked inflammatory effects at both gross and histopathological levels (Vernooy et al. 2001). These effects include tissue destruction and lesion formation in the pulmonary spaces caused by immuno-inflammatory mediator release and immune complex deposition in the alveolar wall (Rennard 1999). The major cellular mediator in acute sepsis is the neutrophil (PMN). Indeed a massive acute neutrophilia is a characteristic pathological indicator of bacterially induced sepsis and inflammation (Takizawa 1998). Such local effects of LPS instillation are paralleled by systemic changes *in vivo* (Traber et al. 1992), changes which are broadly dose-dependent, and of such significant import as to precipitate pulmonary failure and death in subjects exposed to sufficient quantities (Moore & Standiford 1998).

The molecular basis of the acute inflammatory response to LPS and bacterial invasion is well established and extensively reviewed (Moore & Standiford 1998; Shanley, Warner, & Ward 1995; Toews 2001; Xing et al. 1999; Levine 1995). The innate immune effector arm responsible for the reaction to LPS is initiated via activation of alveolar macrophages. Activated alveolar macrophages release cytokines, a number of which induce division of myeloid precursors in bone marrow thus releasing PMNs into the circulation and causing a characteristic neutrophil leukocytosis (Standiford et al. 1990). The circulating PMN population is then localised via a number of chemotactic factors released by the alveolar macrophages and epithelial cells that draw the neutrophils into the alveolar spaces (Takizawa 1998). Activation of the complement system is concurrent with this PMN induction and serves to increase the phagocytosing properties of the PMNs through the opsinisation of pathogenic material

within the alveolar space (Rabinovici et al. 1992; Worthen et al. 1987) though complement is not a necessary part of this process (Brauer et al. 2000; Cardozo et al. 1991). The neutrophils are also potent producers of chemokine and cytokine mediators and thus further potentiate the inflammatory response. The most important cytokine mediators of acute inflammation in its initial phase are the interferons (Heremans et al. 2000), tumour necrosis factor (TNF) α (Xing et al. 1993; Rochemonteix-Galve et al. 1991; Remick et al. 1990) and interleukins (IL) 1β (Rochemonteix-Galve et al. 1991) and 6 (Benbernou et al. 1992; Callery et al. 1991) which are produced by epithelial cells and a number of immune cells as well as neutrophils. The chemokine interleukin 8 is also an important factor in the neutrophil infiltration during acute inflammation (Harada et al. 1994; Koyama et al. 2000; Lin et al. 1994; Standiford et al. 1990). In limiting the inflammatory response the anti-inflammatory interleukin 10 plays a central role and is considered the major factor in the negative feedback loop preventing self-harm by blocking the hyper-inflammatory reaction (Inoue 2000; Kwong et al. 1998; Lo, Fu, & Cryer 1998; Raychaudhuri et al. 2000; Shanley, Vasi, & Denenberg 2000).

IL1β activates lymphocytes and mediates tissue destruction (Dinarello 1994; Ulich et al. 1991) whilst TNFα improves IgG infiltration to pulmonary spaces as well as improving traffic of immune cells into tissue (Ulich et al. 1991; Zhao et al. 2003). Systemic effects of IL1β include pyrexia and increased production of IL6 (Dinarello 1994). TNFα also induces pyrexia and the mobilisation of metabolites and can cause shock at high levels (Xing et al. 1999). Following the production of IL1β and TNFα by both airway and immune cells, and partly mediated by their presence, IL6 is produced by epithelial cells and alveolar macrophages (Crestani et al. 1994). IL6 is a potent activator and chemoattractant of lymphocytes and neutrophils (Dentener et al.

2000), increases antibody production and stimulates IL8 production from alveolar macrophages as a co-factor with IL1β and TNFα (Dinarello 1994; Koyama et al. 2000; Lin et al. 1994). Systemically IL6 induces pyrexia and up-regulates the production of acute-phase protein production (Dentener et al. 2000). IL8 is the major neutrophil chemoattractant and acts to improve access of effector cells to the affected tissues as well as increasing binding of β_2 integrins (Harada et al. 1994; Yamamoto et al. 1998). Neutrophils are activated by the combined effects of TNFα and IL8 and they in turn produce both factors as well as IL1β forming a positive feedback loop that persists and continues to be amplified as long as the tissue continues to be stimulated by the LPS. Such progressive amplification and can result in systemic organ failure and shock (Matthay, Uchida, & Fang 2002; Muller 1992).

As the inflammatory response progresses the combined effects of the above cytokines, along with several other molecular mediators, including chemokines, complement and other immune factors result in influx and activation of T helper cells and the switch from an acute, innate response to a long-term adaptive response. This switch is mediated by a number of factors with IL10 predominant in down regulating the acute phase response (Thomassen, Divis, & Fisher 1996). Type 2 T helper cells (TH2 cells), essential for antibody-mediated immunity and which are attracted by IL6 production are the major producers of IL10, and thus act as key negative regulators of the acute phase response (Inoue 2000; Mocellin et al. 2004; Raychaudhuri et al. 2000). IL10 inhibits the activation of alveolar macrophages and is a potent inhibitor of cytokine release, in particular the macrophage associated cytokines such as IL6, IL8, IL1β and TNFα (Kwong et al. 1998; Lo, Fu, & Cryer 1998; Raychaudhuri et al. 2000; Shanley, Vasi, & Denenberg 2000; Thomassen, Divis, & Fisher 1996).

The acute, non-adaptive immune response to endotoxin is mediated by a wide variety of molecular factors. The underlying network of molecular immune effectors controls and directs the cellular components of both the innate and the adaptive immune systems.

The sheep as a model for LPS-mediated direct lung injury

In human subjects, local lung segmental instillation of LPS (1-4 ng/kg) induced a focal inflammatory response characterised by an early phase (2h to 6h) influx of neutrophils and elevated cytokine and chemokine levels in bronchoalveolar lavage fluid (BALF) (O'Grady et al. 2001). The data demonstrated that endotoxin-induced inflammation in the human lung has a unique qualitative and temporal pattern of inflammatory mediator and cell marker expression compared with systemic endotoxin responses.

Brogden et al (1984) evaluated the clinical and pathologic response to local bronchoscopic instillation of *E. coli* and *Pasteurella haemolytica* LPS in sheep lungs (Brogden, Cutlip, & Lehmkuhl 1984). The instillation caused an inflammatory response characterised by both local and systemic neutrophilia and macroscopic effects including fibrinopurulent inflammation, haemorrhage, oedema and hyperaemia, with concomitant focal necrosis in the sheep instilled with *P. haemolytica* LPS. The effects of the LPS deposition were comparable to those seen in association with gram-negative pneumonia.

The results of local lung instillation of LPS in sheep bear favourable comparison with those seen in human subjects and are consistent with the accepted picture of inflammatory response to endotoxin.

As such an ovine model of direct lung injury based on local lung instillation of LPS holds relevance as a primary insult with which to explore the concept of such local

insult inducing a shift in whole organ susceptibility, or a 'Remote response' to local lung injury. The strategy of challenging one area of the lung and comparing the response in that area with a further 'control' area mimics that commonly employed in large animal models and is frequently referred to as a segmental approach.

Modelling responses at the lung segmental level

Experimental large animal models of lung inflammation frequently use segmental challenges to model diseases such as COPD (Hiemstra, van Wetering, & Stolk 1998), asthma (Bice, Seagrave, & Green 2000), ARDS (Pompe et al. 1996; Forsgren et al. 1990; Sielaff et al. 1987), emphysema (Susskind et al. 1985) and pneumonia (Monton & Torres 1998). The lungs of large mammals are lobar and segmented, with each segment being anatomically distinct from its neighbours and named to correspond to the major airway entering it. Segmental challenge models in large animals assume segmental independence in lung reactions such that each anatomically separate segment reacts in an independent manner to challenge. This model allows for each lung to act as its own control, thus reducing the need for control groups and hence reducing the numbers of animals used in any given experiment. Segmental independence also allows for investigations with three or more treatment groups and controls within individual animals. This segmental independence in the lung has been shown phenotypically (Collie et al. 2001) through gross histopathological study and analysis of immune cell content of bronchoalveolar lavage fluid, but has not been established at the molecular level. Thus, the study of remote responses has broader implications in validating or disproving the assumption of segmental independence in these models. Although lacking formal proof in the lungs, the precedent for local events influencing whole organ physiology is well established in other organ systems.

Remote responses

A remote response is henceforth defined as evidence of change as a consequence of direct perturbation of normal tissue processes in a spatially disparate location in the same organ.

Lung

Prior data suggests that, at least at the phenotypic level, remote responses to direct local lung insult are not commonly observed. The majority of data concerns larger animal models where the benefits of a larger lung facilitate bronchoscopic intervention and the design of protocols relying on the assumption of lung segmental independence both with respect to delivery of substances and the respective segmental responses.

Indeed in sheep Rawn et al (2000) in investigating the regional cellular and histopathological effects of local lung antigen challenge concluded that the cellular phenotypic response of the lung was compartmentalized to the area or segment challenged (Rawn et al. 2000). These results are in agreement with several others in this species where a lack of change manifest at the phenotypic level in control or naïve lung segments is taken as evidence that segmental responses in treated lung segments remain focally localised and independent, and neither responsible for or influenced by changes in other lung segments (Collie et al 2001, Emerson et al 2003, Bischoff et al 2003).

Although lacking formal proof in the lungs, the precedent for local events influencing whole organ physiology is well established in other organ systems.

Very few studies have deliberately investigated remote responses to inflammatory stimulus as a primary investigative aim, however a number have elucidated organ-

wide changes by serendipitous happenstance as part of a local inflammatory or traumatic challenge model (Hoang et al. 1997; Sun et al. 1998; Hanatani et al. 1998). Almost all of the remote responses elucidated thus far are sub-phenotypic, and often the effects are subtle and their impact poorly understood. Remote changes in gene expression are not consistently quantitatively related to distance from the focus of stimulus (Omura et al. 2000; Yoshiyama et al. 1997) and many appear to be regulated in an entirely independent manner from the genes induced locally by a challenge. The evolutionary impetus behind the remote response to a localised stimulus could be as a priming mechanism to prepare organs for further systemic insult (Zhai, Futrell, & Chen 1997) or as a protective downgrading response to avoid systemic organ-wide inflammation with concomitant damage to host organs (Keyvani et al. 2000).

Brain

A major source of evidence for remote genotypic responses following local challenge is the rat brain. A 1997 study by Zhai et al found significant increases in expression of inflammatory cytokine genes (TNFα and IL1β) in the contra-lateral brain and remote ipsilateral tissue following middle cerebral artery occlusion (MCAO) in rats (Zhai, Futrell, & Chen 1997). In a similar study using quantitative RT-PCR Jander et al (2000) demonstrated up-regulation of IL1β and TNFα in the remote ipsilateral sections of the brain following MCAO, though not in tissue from contra-lateral sites (Jander, Schroeter, & Stoll 2000). A number of studies in the rat brain have shown remote regulation of neurotrophic factors following traumatic brain injury (Truettner et al. 1999; Oyesiku et al. 1999; Hicks et al. 1999). These studies used fluid percussion (Hicks et al. 1999; Truettner et al. 1999) or impact trauma (Oyesiku et al. 1999) in order to injure the brain. Various molecular detection methods were employed to detect changes in neurotrophic factor expression in the brain. All three

studies showed remote contra lateral up-regulation of brain-derived neurotrophic factor (BDNF). Oyesiku et al (1999) also found significant changes in expression of neural growth factor (NGF), cerebral neurotrophic factor (CNTF) and its receptor as well as tyrosine kinases A and B (trkA, trkB) (Oyesiku et al. 1999) in the absence of any detectable phenotypic change. Truettner et al (1999), using non-quantitative methodology, demonstrated similar changes in BDNF and NGF (Truettner et al. 1999). Hicks' study found no remote change in trkB expression (Hicks et al. 1999). Kinouchi et al published two studies in 1994 investigating expression of immediate early genes following MCAO in the rat. The first study found increased expression of NGFI-A remote from the infarcted region (Kinouchi et al. 1994b) using *in situ* hybridisation (ISH), whilst the second found induction of c-fos, c-jun, junB and hsp70 in various regions remote from the site of infarction (Kinouchi et al. 1994a). This expression of immediate early genes appeared to be a 'spreading' response to the infarction, with expression peaking first in the infarcted region, and then increasing at later time points in the remote regions. These studies are supported by findings in the mouse brain by Liu et al (2000) in which increased expression of c-jun was demonstrated in the remote contra lateral regions of the brain (Liu et al. 2000). Keyvani et al (2000) demonstrated significant two-fold reduction in expression of proteosome C2 in contra lateral rat brain following focal brain ischemia (Keyvani et al. 2000). Proteosome C2 is involved in protein turnover in the brain and the authors postulated that the reduction in remote sections of the brain represents a protective reaction following localised ischemia. Similarly, increased expression of COX-2 bilaterally from ischemic lesions was demonstrated in human cadaver brain specimens using immunohistochemistry and northern blotting techniques (Sairanen et al. 1998).

Heart

A number of studies in the heart have found remote responses post-myocardial infarction. Increased level of messenger RNA for β-MHC, α-skeletal actin and sodium-potassium exchanger genes occur in remote, non-infarcted myocardium (Yoshiyama et al. 1997). The pattern of gene expression differed to that occurring in the infarcted tissue and adjacent non-infarcted tissue, suggesting discrete regulatory patterns in spatially disparate areas of the organ. In 1998 the same group reported altered expression of α-skeletal actin, atrial natriuretic peptide (ANP) and collagen I and III in remote non-infarcted heart tissue, a pattern of gene expression that differed from that seen in the infarcted areas (Hanatani et al. 1998). Long-term (4 months post infarction) alterations in gene expression in non-infarcted myocardium of ATPase and sodium-potassium exchanger genes were also highlighted by this group with the authors suggesting that these genes are expressed remote from the site of infarction as a compensatory reaction to tissue damage, or as a result of tissue remodelling throughout the organ (Omura et al. 2000). These studies also demonstrated that a remote response can occur at the molecular level with no concurrent phenotypic change, and that the characteristics of the remote response can be separate and distinct from that seen at the site of injury or stimulus. Further, remote expression of endothelin-1, ANP and insulin-like growth factor-1 (IGF-1) was demonstrated in post-infarction rat hearts by RT-PCR with the expression patterns linked to the size of the infarcted area (Loennechen et al. 2001). A further experiment in rats by Sun et al (1998) demonstrated remote expression of angiotensin II and its receptor (ACE) as well as transforming growth factor β_1 (TGFβ) following myocardial infarction(Sun et al. 1998). This remote expression was associated with low levels of fibrosis, and was thus implicated in the repair process in the heart. These studies indicate that, in the

heart, there is clear evidence for a molecular response to injury, which is independent of the response in the directly challenged area of the organ.

Joints

Various studies in rats have demonstrated that an induced monoarthritis generates an inflammatory response in the bilateral joint without involvement of the inducing factor or concurrent inflammation in ipsilateral joints (Kidd et al. 1995; Decaris et al. 1999; Lombard et al. 1999). Various mechanisms have been suggested. The predominant theory regarding induction of a bilateral inflammatory response to unilateral stimulus is that the immune and inflammatory cascade is mediated via neurogenic signalling in a symmetrical fashion (Tseng et al. 2001; Decaris et al. 1999; Kidd et al. 1995; Tsuruoka et al. 2003; Lombard et al. 1999) with further evidence suggesting that this is mediated by the NF-kB cascade (Miagkov et al. 1998). It is of note that, in rabbits, adenovirus-mediated gene transfer of IL 1 and TNFα receptors to knee joints with experimentally induced arthritis resulted in decreased inflammation in both the directly treated joint and in the bilateral knee joint, with this effect appearing to be independent of direct infiltration of the adenovirus vector (Ghivizzani 1998). These results were at the phenotypic level and did not include molecular characterisation.

Kidneys

A further study involving gene delivery and expression was conducted by Kluth et al (2001) in rats with experimentally induced glomerulonephritis (Kluth et al. 2001). Following unilateral injection of macrophages transfected with adenovirus expressing IL 4, inflammation was attenuated in both the treated kidney and the bilateral kidney.

The mechanism by which organs respond in a bi-lateral or stimulus-remote manner is unknown; however a number of reviews and research publications have hypothesised possible mechanisms. Review articles by Downing (Downing & Miyan 2000), Barnes (Barnes 1996) and Scott (Scott, Lam, & Ferrell 1994) have all postulated that the immune system communicates with the neural network via a number of neuroeffector mechanisms. In particular the neurogenic pathways have been implicated in the regulation of inflammatory and immune responses via effector molecules such as substance P. This network has been shown to be active in the lungs as well as a number of other organs and systems (Barnes 1996) and direct disruption of the action of substance P in the lung has been shown to attenuate immune complex deposition in the mouse (Bozic et al. 1996). Reviews of lung innervation have also noted the links between neuronal pathways in the lung and inflammatory responses (Belvisi 2003), and have postulated that a bilateral response to stimuli would be consistent with the known models of neurogenic injury (Perez Fontan 2002).

The Hypothesis

That the lung will mount a sub-phenotypic response remote to the site of local lung injury and that this response will be amenable to characterisation at the molecular level.

In order to challenge the hypothesis sensitive tools to characterise any induced response are required. In the sheep such analysis currently founders on a relative dearth of species-specific probes and reagents. The combined uses of microarray and suppression subtraction hybridisation library techniques have already been shown to be effective as tools for analysis of lung disease in rodent models (Dougherty & Geschwind 2002) and human cell line models (Yang et al. 1999). Both of these techniques are reviewed below.

Addressing the Hypothesis

Model validation

The robustness of the proposed ovine model of local lung segmental challenge with LPS in eliciting a focally discrete inflammatory response, at least at the phenotypic level, requires initial validation.

Addressing the hypothesis at the single gene level

A conventional 'single-gene' approach to characterising the inflammatory response in directly challenged and remote lung segments will provide the means to address the hypothesis using a limited number of currently available and well-characterised species-specific molecular probes.

Gene discovery and microarray development

Conventional molecular analysis is necessarily limited to those genes that have already been characterised in the ovine genome and as a consequence effectively narrows the window on events. In the absence of tools to examine global responses in ovine gene expression a process of gene discovery and array assembly is required to fully characterise the nature and extent of a remote response in the lung.

Addressing the hypothesis using microarray gene profiling

The output from the array will furnish a snapshot of global transcriptional activity in epithelial cells derived from the directly challenged and remote lung segments.

A central component to challenging the hypothesis in the above manner is the development of a cDNA microarray platform that will combine currently available

cDNA resources with respect to a closely homologous species (bovine) with an organ-specific selection of clones from the species in question.

The combined uses of microarray and suppression subtraction hybridisation library techniques have already been shown to be effective as tools for analysis of diverse diseases and models including retinal disease in cattle (Schulz et al. 2004), paramyxovirus infection *in vitro* (Munir et al. 2004), nasopharyngeal carcinoma in humans (Zhang et al. 2003) and breast cancer in human cell line models (Yang et al. 1999). Both the SSH and microarray techniques are reviewed below.

Microarrays

Overview

Microarrays are a miniaturised format in which multiple array elements (cDNA, oligonucleotides, proteins etc.) are bound as 'probes' to a solid substrate and interrogated with a labelled target. Microarray technology has its basis in solution hybridisation methods in which kinetic studies of the hybridisation of mRNA pools with radioactively labelled cDNA were undertaken (Bishop & Smith 1974). These solution hybridisation methods were superseded by hybridisations based on filter blotting techniques, defined first as Southern blots (Southern 1975). The theory underpinning these techniques is that a labelled probe will bind to a target (in nucleic acid probes this target would be a homologous sequence to the probe), allowing the target to be detected. DNA microarrays work by binding a nucleic acid probe to a solid support, such as glass slides or nylon filters, and labelling the target DNA or RNA. The labelled nucleic acid is then hybridised to the probe, stringency washes used to remove excess and the label detected in order to determine which probes have been bound and to what extent (Southern, Mir, & Shchepinov 1999).

DNA microarrays have become increasingly miniaturised due to the use of robotic printing methods. Nucleic acid spots of 100 – 300 μm diameter are deposited onto the solid array supports (Cheung et al. 1999). The support substrates themselves have also aided greater miniaturisation with early nylon filters giving way to coated glass slides (Lander 1999). The direct deposition method of printing microarrays using high precision robots can be divided into two categories: the more widely used cDNA microarrays in which fairly long (0.6 – 2.4 kilobases in length (Duggan et al. 1999)) PCR products are deposited onto slides (Schena et al. 1995); and oligonucleotide microarrays in which much shorter (25-100 bases (Hacia et al. 1999)) single-stranded sequences are attached to a prepared glass surface. These direct printing methods were pioneered by Patrick Brown at Stanford University (Schena et al. 1995) and function by using robots to acquire the nucleic acids in nanolitre - microlitre quantities, transferring the sample to pre-designated co-ordinates on the slide and depositing them using miniaturised pins (Cheung et al. 1999).

Both of these techniques use labelled nucleic acids as a target, the label usually being a nucleic acid-linked fluorochrome, though others, including radioactive labels, are also used. The labelled target is hybridised to the microarray for between 8 and 24 hours (Cheung et al. 1999) after which the array is washed and then scanned to detect the level of signal from each gene or control 'spot.' The scanned image is processed electronically to determine the signal and background intensities for each spot. Once the image data has been acquired the results are scaled and normalised prior to analysis.

Microarray technology allows for the analysis of expression of many thousands of genes simultaneously, providing a 'snapshot' of a cell's transcriptome at any given point in time. This massively parallel investigation technique is potentially

very powerful and is ideally suited to analysis of complex interlinked or cascade effects within tissues. The technique is also highly applicable to situations in which little is known concerning the effects of given treatments or procedures as these can be screened and further investigated should they be found to be of significant import. Microarrays therefore appear to be an ideal method for investigating remote response genetic effects, as these are likely to be sub-phenotypic and complex in nature.

Gene Discovery

Overview

The total number of sequenced and characterised genes in sheep is under 4000, representing approximately 1% of the total genome. There is only one large scale genome mapping project currently identified for sheep in the Genomes Online Database (GOLD) (Bernal, Ear, & Kyrpides 2001; Kyrpides 1999), and this project is concerned with identification of quantitative trait loci in contrast to the full-length sequencing and characterisation projects undertaken in bovine, human and rodent species.. This lack of available genetic data hinders research into sheep genomics, and particularly microarrays, as there are far fewer targets available for investigation. This deficiency is exacerbated by the fact that a large proportion of the genes that are available are commonly expressed maintenance (or housekeeping) genes that are unlikely to be significant interest in a disease model. In order to successfully construct an ovine microarray for use in the lung it is therefore necessary to generate a number of 'new' ovine sequences that are known to be expressed in lung tissue. Various methods of gene discovery are available ranging from genome sequencing to tissue-specific library construction.

It is preferable to utilise gene discovery techniques that both produce lung specific results and encompass genes that are expressed at all levels. In order to find genes expressed at low levels in the lung a suppressed subtracted library approach is appropriate, whilst finding the more highly expressed genes in lungs requires a broader screening process. Various screens are of potential use in discovering medium to high expression genes, including genomic library screening and heterologous screens against well characterised and closely related species to sheep – in particular cattle. Heterologous screening carries the advantage that the selected heterologous genes are more likely to be well annotated and characterised than the clones available in a genomic library.

Review of SSH

Subtractive Suppressive Hybridisation (SSH) is a PCR-based technique that allows RNA messages present in a tester population but absent in a driver population to be isolated whilst at the same time normalising the quantities of the final sequences in order to make less abundant species more accessible (Diatchenko et al. 1996). The method works by taking two RNA populations termed 'tester' and 'driver' and reverse transcribing these to cDNA. The cDNA populations are then restriction digested and the tester population is adaptor ligated using two adaptors. A first hybridisation is used with excess driver cDNA in order to equalise the populations and enrich for differentially expressed genes, followed by a second hybridisation which generates PCR templates from the differentially expressed sequences. Two rounds of PCR are used to amplify the templates and reduce background, the first with adaptor primers and the second with nested primers to further eliminate non-differentially expressed sequences (Diatchenko et al. 1996). The procedure is outlined diagrammatically in Figure 2.1.

After the SSH, the resulting PCR products are cloned into *E. coli* and grown in 96-well plates. This library can then be used to generate PCR products directly from the clones, and these are then ready to be printed onto microarray slides. This method has been used in order to generate subtracted libraries in humans for testis (Diatchenko et al. 1996) colorectal cancer (Bustin et al. 2001), gastric cancer (Carvalho et al. 2002) and cell lines including colon cancer (Bertram et al. 1998), haematopoietic (Beyer-Sehlmeyer et al. 1999) and cervical and prostate lines (Endege et al. 1999). It has also been used in strains of *E. coli* bacteria (Janke et al. 2001). The SSH technique has also been used to generate a microarray for ER-negative breast cancer cell lines (Yang et al. 1999) which clearly demonstrated that the sequences isolated by SSH were highly differentially expressed in the tester population demonstrating the techniques suitability for use in generating novel microarrays.

Clearly the idea of organ-wide genotypic change is one that requires further investigation in order to fully characterise the responses to insult and their effects. A quantitative, structured and parallel investigation of remote responses is desirable not only in relation to inflammatory lung diseases such as ARDS but also to analyse the possible effects that such responses may have on segmental studies that rely on the assumption that there is little or no general effect resulting from local insult. In systems used to model disease, particularly inflammatory disease, these regional effects could potentially highlight novel avenues of intervention or prophylaxis.

Summary

It is clear from the published body of literature that a number of organs demonstrate a remote response to localised inflammatory stimulus. It is also clear that the existence of a remote response in lungs needs to be evaluated both in order to establish the biological basis of pulmonary inflammation and to address a number of practical

issues in pulmonary medicine such as the potential mechanism of ARDS pathology and the validation of the segmental challenge model of lung inflammation. The ideal platform for the investigation of subtle molecular regulation in the lung is the microarray, and the lack of available ovine microarrays requires the generation of novel array elements. The experimental plan is to produce a subtracted, suppressed library representing gene sequences unique to or differentially expressed in lung tissue. Additionally a homology search of publicly available cattle libraries for clones with high levels of homology with known ovine sequences will be made and those clones showing high levels of homology (>70%) selected and combined with the SSH library clones. PCR products will be amplified from the combined clone set and printed as a microarray. This array will be used to probe the expression of genes from a remote segment of ovine lung compared with a segment directly subjected to a significant inflammation with bacterial LPS, and the differences in expression analysed between the segments in a group of out bred sheep (n=12). In addition to answering the hypothesis this experiment will also characterise the inflammatory response to bacterial LPS at the molecular level.

Chapter 2 Materials and methods

Overview

One lung segment from both the right and left lung of each animal was selected on bronchoscopic examination and its position carefully mapped. One segment, designated 'direct', was selected for LPS instillation and the segment from the contra lateral lung, designated 'remote', used to assess the whole organ response. 1 mg E. coli O1:B6 LPS (Sigma-Aldrich; Dorset, UK) in 5ml normal saline was used, selected on the basis on phenotypic changes seen in previous ovine model investigations by members of the laboratory team (personal communication, S.Tait). Bronchial epithelial cells and BAL fluid were collected from each segment by bronchial brushing and then bronchoalveolar lavage not less than two weeks prior to instillation of LPS and at 6 hrs post instillation. Following this collection the sheep were killed and necropsy samples taken for histopathological analysis.

The BAL fluid (BALF) samples were analysed for total and differential cellular content in order to quantify the level of phenotypic change as a consequence of instillation. Haematological profiles were assessed in blood samples collected prior to and subsequent to instillation and, in conjunction with rectal temperature measurements, as indices of systemic effect of the LPS instillation. Lung samples taken at necropsy were assessed by veterinary pathologists in order to provide a qualitative index of the gross and histopathological changes in lung segments occurring as a consequence of instillation. The results from the phenotypic analysis of the samples are presented in chapter 3.

The brushed epithelial cells were visualised by microscopy to analyse content, lysed, total RNA extracted and the sample divided into two aliquots. The cellular content of

the brushing samples were uniformly over 90% epithelial cells, with the remainder almost entirely consisting of monocytic cells, approximately 35% of samples contained significant numbers of erythrocytes in addition. Half of each sample was used for real-time quantitative RT-PCR analysis (chapter 4) and half used for amplification and labelling prior to hybridisation to custom-made ovine lung microarrays (chapter 6).

The requirement for a novel microarray to profile transcriptional activity in response to LPS instillation necessitated a process of gene discovery and selection. The gene discovery effort was focussed around the identification and selection of homologous bovine genes in a commercially available cattle library and the creation of a novel lung-specific library. The lung specific library was constructed using a commercial technique called Suppressive Subtractive Hybridisation (SSH), and resulted in a collection of clones representing a normalised and subtracted ovine lung population. These two collections of clones were combined and their inserts used to produce amplified PCR products that were, in turn, printed onto slides to make a new cDNA microarray. The details of the homology searching, gene discovery and SSH library construction are presented in chapter 5.

Gene discovery

BLAST

Homology searches were performed to find homologs between known ovine gene sequences and the clones in the cattle libraries MARC 1, 2, 3 and 4 and BARC 5 (http://www.ark-genomics.org/resources/cattle.php). Searches were conducted using the Basic Local Alignment Search Tool (BLAST), a public access program from the National Centre for Biotechnology Information in Bethesda, MD, USA. (Altschul et

al. 1990). This program uses a statistical algorithm to find homologies within genes stored in the GenBank database. The searches were carried out using an expect value of $1x10^{-7}$ and taking positive results as those with a bit score over 100 that showed greater than 70% homology.

SSH

Sample collection

Tissue samples from heart, lung, liver, kidney, brain and skeletal muscle were taken at necropsy from ex-sanguinated sheep, stored in 5 volumes of RNA*later* at 4°C overnight and then at -20°C long-term. Six crossbred sheep were used (2 male and 4 female; mean age 3.8 years [range 1-6]). Samples were taken from multiple sites throughout the various tissues.

cDNA subtraction

The SSH was completed using the PCR-Select cDNA subtraction kit (Clontech, Palo Alto, USA) according to the manufacturer's instructions. Each experimental subtraction used 2 µg mRNA from lung as tester and 2 µg mRNA from the pooled heart, muscle, liver, kidney and brain samples as driver. Positive controls comprised the RNA provided in the kit and negative controls used water as tester and pooled RNA as driver. The cDNA subtraction was divided into four main parts, double stranded (ds) cDNA preparation, digestion and ligation, hybridisation and finally amplification.

The production of ds cDNA began by incubating 2 µg mRNA with 1 µl cDNA synthesis primer (10 µM) at 70°C for 2 minutes, and then on ice for two minutes – to this were added 2 µl 5 X first-strand buffer, 1 µl dNTP mix (10 mM) and 20 units AMV reverse transcriptase to a total volume of 10 µl. This was incubated at 42°C for 90 minutes in an air incubator and the reaction terminated by placing it on ice. Second strand synthesis was achieved by adding 16 µl 5 X second strand buffer, 1.6 µl dNTP mix (10 mM) and 4 µl 20 X second strand enzyme cocktail to the first strand mixture in a total volume of 80 µl. The solution was incubated at 16°C for 2 hours after which

2 µl (6 units) T4 DNA polymerase was added and the reaction incubated at 16°C for a further 30 minutes. The reaction was terminated by adding 4 µl 20 X EDTA/glycogen mix. The cDNA was isolated by adding 100 µl phenol:chloroform:isoamyl alcohol (25:24:1) and then centrifuging. The aqueous layer was taken and 100 µl chloroform:isoamyl alcohol (24:1) added. This was centrifuged and the aqueous layer taken. DNA, precipitated by adding 40 µl NH_4OAc (4 M) and 300 µl ethanol (95%), was pelleted in a centrifuge and dissolved in 50 µl water. 6 µl of this was stored for analysis at -20°C.

The remaining ds cDNA was Rsa I digested by adding 5 µl 10 X Rsa I restriction buffer and 1.5 µl Rsa I (10 U/µl) and incubating at 37°C for 90 minutes. 5 µl was stored at -20°C for later analysis, the remaining reaction was terminated and DNA purified as before, though at half the volumes previously stated. The final pellet was dissolved in 5.5 µl water, stored at -20°C and used as the driver cDNA. 1 µl of the driver cDNA was diluted with 5 µl water. Adaptor ligation proceeded as follows: 2 µl of the diluted driver cDNA were put in 2 separate tubes (1-1 and 1-2) and 2 µl 5 X ligation buffer and 400 units T4 ligase added to each sample. After adding 2 µl adapter 1 (10 µM) to tube 1-1 and 2µl adapter 2R (10 µM) to tube 1-2, water was added to a final volumes of 10 µl. 2 µl of each of 1-1 and 1-2 were mixed to make an unsubtracted tester control tube labelled 1-c. All three solutions were incubated at 16°C overnight and ligation terminated by adding 1 µl EDTA/glycogen mix. Heating at 72°C for 5 minutes inactivated the ligase. 1 µl of the unsubtracted tester control was diluted into 1 ml water and stored at -20°C.

For the first hybridisation reaction 2 mixtures were made. Mix one contained 1.5 µl Rsa I digested driver cDNA, 1.5 µl adapter 1-ligated tester cDNA and 4 µl 4 X hybridisation buffer. In mix two the adapter 1-ligated tester was replaced with adapter

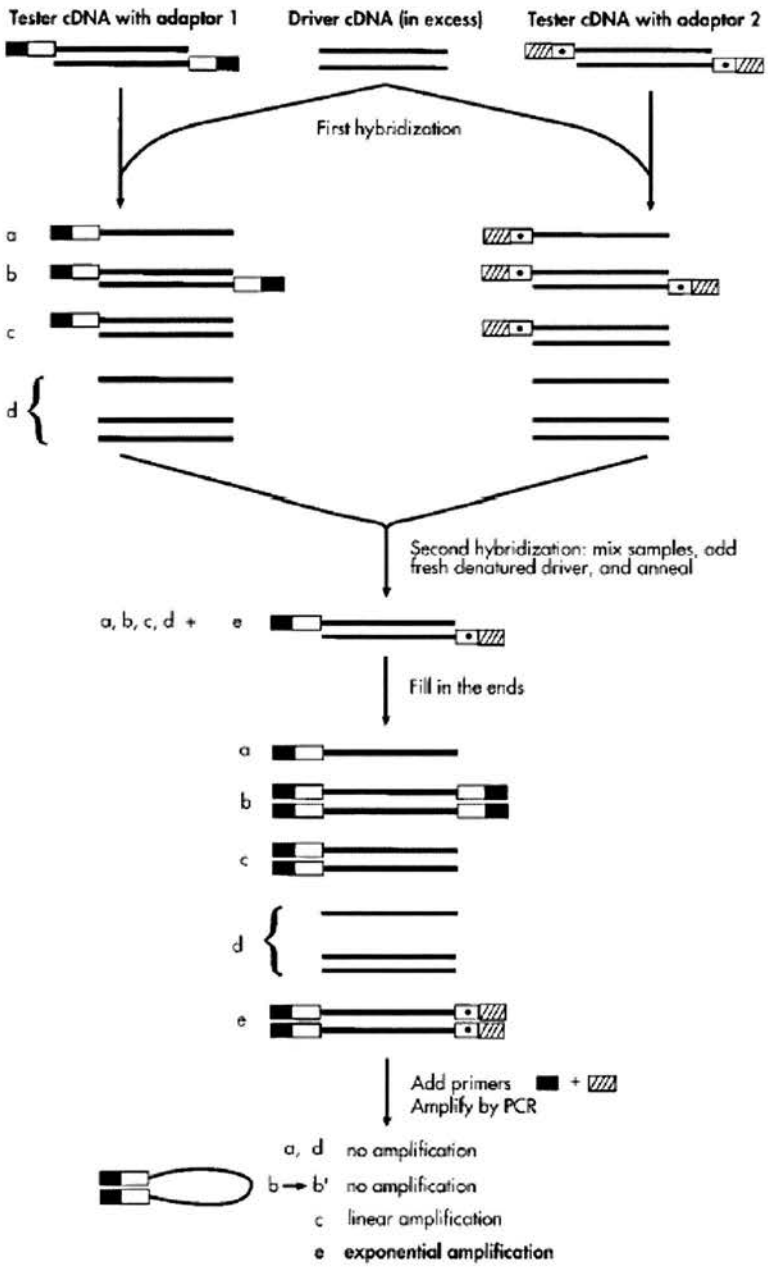


FIG. 1. Scheme of the SSH method. Solid lines represents the *Rsa*I digested tester or driver cDNA. Solid boxes represent the outer part of the adaptor 1 longer strand and corresponding PCR primer P1 sequence. Shaded boxes represent the outer part of the adaptor 2 longer strand and corresponding PCR primer P2 sequence. Clear boxes represent the inner part of the adaptors and corresponding nested PCR primers PN1 and PN2. Note that after filling in the recessed 3′ ends with DNA polymerase, types a, b, and c molecules having adapter 2 are also present but are not shown.

Figure 2.1: This diagram is taken directly from the original paper describing the SSH procedure by Diatchenko et al. It graphically illustrates the SSH procedure, and includes the summary accompanying the diagram from the original publication {Diatchenko, 1996 DIATCHENKO1996 /id}. The procedure described is exactly as presented in the manufacturer's protocol and was followed in this study.

2R-ligated tester. The reactions were incubated at 98°C for 90 seconds and then at 68°C for 8 hours. The second hybridisation step began by incubating 1 µl driver cDNA with 1 µl 4 X hybridisation buffer in 4 µl at 98°C. This solution was then mixed with hybridisation samples 1 and 2 from the first hybridisation step and the mixed samples incubated at 68°C overnight. 200 µl dilution buffer was added and the sample heated at 68°C for 7 minutes before storing at -20°C prior to the PCR step.

To 1 µl of each diluted cDNA (hybridised, control or unsubtracted control) the following were added: 2.5 µl 10 X PCR reaction buffer, 0.5 µl dNTP mix (10 mM), 1 µl PCR primer 1 (10 µM) and 0.5 µl 50 X Advantage cDNA polymerase mix (Clontech, Palo Alto, USA) to a total volume of 25 µl. The mixtures were incubated at 75°C for 5 minutes and then subjected to 30 cycles of 94°C for 30 seconds, 66°C for 30 seconds and 72°C for 90 seconds. 8 µl of each reaction was analysed on 2% agarose gel run in TAE buffer for 1 hour at 60V with crystal violet. The part of each lane of the gel corresponding to DNA over 200 base pairs in length was excised and DNA purified from it using the S.N.A.P. UV-free gel purification kit (Invitrogen Ltd.; Paisley, UK). 1 µl of the purified product was added to the following mixture: 2.5 µl 10 X PCR reaction buffer, 1 µl nested PCR primer 1 (10 µM), 1 µl nested PCR primer 2R (10 µM), 0.5 µl dNTP mix (10 mM) and 0.5 µl 50 X Advantage cDNA polymerase mix to a final volume of 25 µl. This mixture was subjected to 15 cycles of 94°C for 30 seconds, 66°C for 30 seconds and 72°C for 90 seconds. 8 µl of each product was analysed on a 2% agarose gel in TAE buffer (1 hour at 60V with crystal violet). The part of each lane of the gel corresponding to DNA over 200 base pairs in length was excised and DNA purified from it using the S.N.A.P. UV-free gel purification kit (Invitrogen Ltd.; Paisley, UK) and then stored at -20°C in preparation

for cloning. This process is illustrated graphically in Figure 2.1 (Diatchenko et al. 1996).

Cloning

PCR products were cloned into chemically competent TOP10F' *Escherichia coli* using the TOPO TA cloning kit (Invitrogen Ltd.; Paisley, UK) according to manufacturer's instructions. Briefly the PCR products were incubated with the TOPO TA vector and salt solution for 20 minutes at room temperature and then on ice. The vector was then incubated with the *E. coli* for 30 minutes on ice and heat shocked for 30 seconds at 42°C. 250 µl SOC medium was added to the cells, which were then shaken at 37°C for 1 hour. The transformed cells were plated onto ImMedia (Invitrogen Ltd.; Paisley, UK) agar plates (commercially prepared with kanamycin and IPTG) and incubated overnight at 37°C. The remainder was grown overnight in 5 ml sterilised and filtered LB broth (Invitrogen Ltd.; Paisley, UK) in 50 ml falcon tubes until cultures were confluent. Blue vs. white colony counts were made to analyse cloning efficiency. This screen sensitively and specifically analyses both insertion of a DNA fragment into the plasmid, and transformation of the plasmid into the *E. Coli* bacterial cell. Each plasmid contains a polylinker site located at the beginning of the coding sequence of the gene coding for β-galactosidase. The polylinker site contains a variety of restriction sites, and is 54 bases in length (and thus in-frame), and code for 18 amino acids that do not affect the enzyme's activity. Insertion of a DNA fragment into this polylinker site disrupts the expression of the β-galactosidase thus preventing its expression when a DNA fragment is successfully inserted into the plasmid loop. B-galactosidase hydrolyzes IPTG (present in the growth medium) and releases an insoluble blue dye, thus disruption of this gene causes the colonies to be white, whilst those with plasmids containing no insert

present as blue, allowing selection of bacterial colonies containing inserts. The presence of a kanamycin resistance gene in the plasmid used allows selection of bacteria which have been successfully transformed due to the presence of kanamycin in the growth medium. Thus any bacteria growing on this medium have a plasmid transformed into them, and those that are white have a DNA fragment inserted into that plasmid.

Cultures were stabilised by addition of 15% sterile glycerol and stored at -80°C. Cultured libraries were titrated by serially diluting aliquots of stored library and growing on ImMedia (Invitrogen Ltd.; Paisley, UK) agar plates containing kanamycin and IPTG overnight at 37°C and then counting the numbers of colonies present at each dilution to determine the titre.

Clone Screening

PCR reactions from the colonies were prepared by picking colonies directly from the agar plate into PCR solution. PCR was performed using 0.5 µl Taq polymerase, 2 µl 10X PCR buffer, 0.5 µl 10mM dNTP mix (all Sigma-Aldrich; Dorset, UK) and 0.5 µl of each forward and reverse M13 primers (MWG-Biotech, Ebersberg, Germany) in total volumes of 20 µl. The solutions were incubated at 94°C for 2 minutes, subjected to 30 cycles of 1 minute at each of 94°C, 55°C and 72°C, and finally incubated for 7 minutes at 72°C. All PCR products were visualised on 2% agarose gels run in TAE buffer for 1 hour at 60V. Products were stored at -20°C.

Sequencing

Plasmid inserts were sequenced using an ABI 3700 sequencer. Plasmids were purified using the Qiagen MagAttract kit (Qiagen; Sussex, UK) according to the manufacturer's protocol. Plasmid inserts were PCR amplified using primers corresponding to the pCRII plasmid from the Invitrogen TOPO TA cloning kit (Invitrogen Ltd.; Paisley, UK) and reagents supplied in the sequencing master mix from the Packard MultiProbe II robotic dispenser. PCR was carried out using the following temperature cycles:

96°C - three minutes} One cycle

96°C - 30s, 50°C - 30s } 35 cycles

60°C - four minutes } One cycle

Following amplification, the PCR product was cleaned using an ethanol precipitation method. The PCR products were incubated with 10 µl water (Millipore; Livingstone, UK), 5 µl 125 mM EDTA and 60 µl 100% ethanol for 30 minutes at room temperature and then the precipitate collected by centrifugation at 2500 g for thirty

minutes. The pellets were rinsed by adding 150 µl 70% ethanol, mixing and then centrifuging at 2500 g for ten minutes and removing the ethanol. Dried pellets were resuspended in 20 µl water (Millipore; Livingstone, UK) and the DNA sequenced by the Big Dye method on an ABI 3700 sequencer. Sequence trace files were analysed to remove contaminant vector sequences using the VecScreen database and the BLAST program (Altschul et al. 1990), and then further trimmed to account for low quality sequencing. Sequences were assembled into contiguous alignment groups (contigs) using the LaserGene SeqMan program (DNAStar; Madison, USA). Consensus sequences from each contig alignment were analysed to determine nearest homology with known characterised sequences from the NCBI nr and est databases, TIGR sequence database and EMBL nucleotide and protein databases using various BLAST sub-programs (Altschul et al. 1990). Tentative functional activity was attributed to sequences where possible through literature revision or theoretical protein sequence translation and analysis.

Microarray production

Microarray printing, clone preparation, insert sequencing, RNA amplification, microarray hybridisation and microarray scanning were performed by ARK Genomic, Roslin, UK according to the following methods.

Amplified PCR product derived from the clones selected from the SSH library and MARC/BARC bovine libraries were robotically re-arrayed into 384 well microplates. The PCR products were purified and normalised to a standard concentration. Each product was deposited by a *Bio*Robotics MicroGrid II robot (*Bio*Robotics; Cambridge, UK) onto amino silane coated glass CMT-GAPS slides (Corning International; London, UK) where they were bound by natural cross-linkage (hydrogen bonding occurred between amine groups coated on the slide and phosphate

groups in the DNA backbone.) After printing, the slides were baked to allow covalent bonds to form, fixing the cDNA to the slide. All products were spotted in duplicate in a grid pattern. The layout of the arrays and the sequences and clones that they represent are detailed in Appendix III.

Animals

Twelve commercially sourced crossbred adult sheep were used. The sheep were clinically examined by a veterinary surgeon prior to entering the protocol and declared to be free from significant disease. Anthelminthic treatment was undertaken as part of the pre-screening process to ensure the absence of patent lungworm infection (*D. filaria* and *M. capillaris*). These sheep comprised a representative cross-section of animals, unselected in terms of their biology or underlying pathology. The detailed characteristics of each sheep within the sample are given in table 2.1.

Table 2.1: Characteristics of each sheep used in the study. All male sheep were castrated. All sheep were out-bred, commercially sourced and housed indoors in standard pens in groups. Prior to experimental use the animals were assessed for clinical disease by a veterinarian. Overall sex ratio: 5 female: 7 male, mean age: 3.8 years [range 1-7], mean weight: 52.6 kg [range: 36 – 78]

Table 2.1

Animal	Sex	Age	Weight (kg)	Breed
S41	Female	7	70	Greyface
S46	Female	7	69	Greyface
451	Male	1	41	Texel cross
X26	Female	7	70	Greyface
S9	Female	7	78	Greyface
S12	Female	5	64	Greyface
O631	Male	2	42	Blackface
O681	Male	2	36	Blackface
O687	Male	2	44	Blackface
O693	Male	2	39	Blackface
O757	Male	2	38	Blackface
O861	Male	2	38	Blackface

Anaesthesia

Food was withheld for 12 hours prior to anaesthesia, which was achieved by intravenous administration of a single bolus of thiopentone sodium (Thiovet; Novartis) at a dose rate of 20 mg / kg bodyweight. Thereafter sheep were intubated and anaesthesia maintained using gaseous halothane (2-3 per cent) in oxygen and nitrous oxide (50:50). The sheep were placed in sternal recumbency in a large Plexiglas whole body respirator (internal volume: 388 litres). The proximal end of the endotracheal tube was connected to the anaesthetic circuit through a connector in the wall of the box. Pressure in the box was varied by appropriate connection to a large bellows pump (Cuirass; Cape Warwick, Warwick, U.K.) which induced a sinusoidal tidal respiratory pattern, the rate and magnitude of which could be controlled by adjustment of the pump itself. The magnitudes of pressure fluctuations were adjusted to maintain a tidal volume of 10 ml / kg bodyweight. Respiratory rate was adjusted to maintain end-tidal CO_2 measurements in the range 4.5-5.5% (Oxicap Monitor Model 4700; Ohmeda, Louisville, CO, USA). 1 mg *E. coli* O26:B6 LPS (Sigma-Aldritch; Dorset, UK) was administered at a concentration of 0.2µg / ml, delivered in 5 ml saline.

Sample collection

Bronchoalveolar Lavage (BAL)

A flexible fibre optic bronchoscope (5.3mm OD) (Model FG-16X; Pentax U.K. Ltd.) was advanced and wedged in selected segmental bronchi. The subtended segments were lavaged by installation and withdrawal of two, 20 ml aliquots of normal saline (0.9% NaCl solution). BALF was immediately placed on ice until subsequent analysis. Lavage fluid from each lung lobe was separated into supernatant and cells by

centrifugation at 400 g for 7 min and the resultant cell pellets were washed twice in phosphate buffered saline (PBS).

Brushing

After the bronchoalveolar lavage (BAL) a sheathed biopsy brush (Olympus BC-202D-2010; Keymed, Livingstone, UK) was introduced through the bronchoscope, and the surface of the bronchus entering the segment of interest was brushed vigorously. A movie clip of the brushing procedure is included in appendix II. The brush was re-sheathed and withdrawn. After removal from the bronchoscope, the brush was unsheathed and passed repeatedly through the end of a modified disposable pipette tip into a sterile 1.5-ml centrifuge tube containing 1 ml of RNAlater (Ambion; Huntingdon, UK). The solution was agitated, the brush removed and the cells stored at –80°C in RNAlater (Ambion; Huntingdon, UK) until the RNA purification step.

Systemic monitoring

Prior to each anaesthetic induction rectal temperatures were recorded and venous blood samples (2 x 7 ml) collected into heparinised vacutainer (Fisher Scientific; Loughborough, UK) tubes. One blood sample was submitted to the Veterinary Pathology Unit Clinical Laboratories, Easter Bush Veterinary Centre (EBVC) for routine haematological analysis and the second sample was centrifuged at 2000 g for 20 minutes to separate the blood fractions (red blood cells, white blood cells and plasma). The Buffy coat layer was removed and stored in RNAlater (Ambion; Huntingdon, UK) at 4°C overnight and thereafter at -20°C as archival material for future study.

Histopathology

Lungs were instilled via the airway with 50% neutral buffered formalin (pH 7.0) at an inflation pressure of 2.5 to 3.0 kPa until fixed. 3 tissue blocks were sampled from each lung segment and routinely processed and embedded in paraffin. 4-5 μm sections were cut from the tissue blocks, and mounted and stained with haematoxylin and eosin for histopathological assessment by a veterinary pathologist.

Cell counts

Total cell counts

Cells were counted using a Neubauer haemocytometer and values expressed per millilitre BALF. $1x10^5$ cells were used in the preparation of cytospin slides. The remaining cells were stored in RNAlater (Ambion; Huntingdon, UK) at 4°C overnight, and thereafter at -20°C. Aliquots of supernatant were stored at -80°C.

Differential cell counts

The BAL cell pellet was re-suspended in PBS to a cellular concentration of 10^5 cells / ml. 100 μl of the suspension (10^4 cells) was placed in a cytospin (Thermo Shandon; Runcorn, UK) collection funnel and centrifuged at 300 rpm for 3 minutes. Two slides were prepared for each sample of BALF. Cytospin slides were air-dried and stained in 100% Leishmann's stain for 3 minutes. Slides were then stained in 50% Leishmann's stain for 6 minutes prior to washing and air-drying. The dried and stained slides were cover-slipped using DPX mounting agent. The mean of two differential cell counts was established on the basis of counting 500 cells from each slide. Cells were classified as neutrophils, macrophages, eosinophils, lymphocytes or mast cells according to standard morphological criteria. Cells not classified in the aforementioned groups were recorded as 'other' cells.

RNA purification

Epithelial cells from bronchial brushings were stored in RNAlater (Ambion; Huntingdon, UK) at -20°C. Prior to processing the samples were diluted 1:1 with sterile PBS and centrifuged for 15 minutes at maximum speed (13,000 g) in a microcentrifuge to pellet the cells. The GenElute Mammalian total RNA kit (Sigma-Aldrich; Dorset, UK) was used to extract total RNA from the cell pellet according to the manufacturer's protocol provided. Briefly, supernatant was removed and the cells re-suspended in lysis buffer. The cells were lysed and solubulised by mechanical shearing and vortexing. The lysate was filtered and mixed with 70% ethanol in a 1:1 ratio. This solution was passed through a binding column, which was then washed twice with each of two wash solutions before being eluted from the column using the elution buffer provided. The RNA was then treated with *DNAfree* DNAse in buffer (Ambion; Huntingdon, UK) for 15 minutes at 37°C to remove remaining DNA contamination; the reaction was stopped by addition of buffer and heating at 70°C for 5 minutes. RNA was precipitated using 0.1 volumes 8M LiCl and 2 volumes 100% ethanol and snap-frozen. The RNA precipitate was stored at -80°C.

RNA quality analysis

RNA quality analyses (both pre- and post-amplification) were performed using the Agilent 2100 bioanalyzer (Agilent Technologies; South Queensferry, UK) (Mueller et al. 2000). The bioanalyzer performs gel electrophoresis in the confines of a micro fabricated chip. Electrical voltages are applied to fluid reservoirs by means of individual electrodes, each of which is connected to a separate high voltage power supply. Samples are separated electrophoretically in an entangled polymer solution, similar to capillary electrophoresis. The instrument allows for highly sensitive laser

induced fluorescence detection using an intercalating dye, which is added to the polymer. The bioanalyzer software automatically calculates size and concentration of each separated band and displays the results in real-time.

All chips were prepared according to the manufacturer's instructions. In brief: gel matrix was prepared by adding 25 µl of dye to a gel vial. The gel/dye mixture was filtered through a spin filter. The chip was filled with the gel/dye mixture and 9 µl of gel/dye mixture were filled into the buffer wells. Sample and ladder wells were filled with 5 µl of RNA marker solution before adding 1 µl of ladder and sample in the respective wells. The chip was vortexed and placed in the bioanalyzer for analysis. Fluorescent trace images were translated by the software to provide a numerical RNA integrity score, and a 'gel-like' image indicating RNA integrity in an extrapolated standard RNA denaturing gel format. RNA exclusion was based on visual analysis of the peaks present on the fluorescence electropherogram. Samples which lacked peaks corresponding to the ribosomal RNA content of the sample were considered of insufficient quality for amplification.

RT-PCR

Reverse transcription

The RNA was pelleted from the ethanol solution by centrifugation at maximum speed for 20 minutes and re-suspended in PCR grade water (Sigma-Aldrich; Dorset, UK). The RNA was reverse transcribed using the Superscript II kit (Invitrogen Ltd.; Paisley, UK) according to the protocol provided. Briefly, 10 µl RNA was incubated at 65°C with 1 µl oligo-dT primers and 1µl 10 mM dNTP mix for 5 minutes, and then at 42°C for 2 minutes with 4 µl 5X RT buffer, 1 µl *RNAseout* RNase inhibitor (Sigma-Aldrich; Dorset, UK) and 2 µl dithiothreotol (DTT). 1 µl Superscript II reverse

transcriptase was added and the reaction incubated at 42°C for 50 minutes. The reaction was stopped by heating to 70°C for 15 minutes and the cDNA stored at -20°C.

Quantitative real-time PCR

Each real-time PCR reaction was performed in triplicate in white, hard-shell, skirted 96-well miniplates with optically clear strip-caps (Genetic Research Instrumentation Ltd.; Braintree, UK). Each reaction consisted of 1 µl template cDNA, 0.5 µl forward primer (5 pmol), 0.5 µl reverse primer (5 pmol), 8 µl PCR water (Qiagen; Sussex, UK) and 10 µl 2X SYBR green master mix (Qiagen; Sussex, UK). Primer sequences for each cytokine and the housekeeping gene are shown in Table 2.2. These were selected from published literature based on the similarity of their annealing temperatures. Primers were HPLC purified and produced by MWG Biotech (MWG Biotech; Ebersberg, Germany). Each reaction was performed in a total volume of 20 µl in an Opticon Lightcycler (MJ Research; Reno, USA) using the following cycling parameters: 95°C - 15min, 50 x (94°C – 15s, 52°C – 30s, read, 72°C – 30s, read, 80°C – 5s, read), melting curve (40°C-95°C, read every 0.1°C, 0.2s hold), 72°C – 10 min. These parameters were selected following optimisation reactions in which serial dilutions of primer and template were compared, along with temperatures and durations of the cycling parameters. These optimisation reactions were guided by both published literature in which the primers used were successfully employed, and standard practice. Analysis of the results from these reactions indicated that the parameters used were those that uniformly demonstrated high levels of product formation with minimal contaminant or background interference.

Table 2.2: Details of the primer sequences used in the real-time quantitative RT-PCR cytokine analysis. All primers were derived from previously published studies in sheep.

Table 2.2

Primer	Sequence	Reference
IL1β (+)	CCCATTAATGAAGTGATGGC	
IL1β (-)	CTAGGGAGAGAGGGTTTCCA	(Gohin et al. 1997)
IL6 (+)	GCTTCCAATCTGGGTTCA	
IL6 (-)	CCACAATCATGCGAGCCG	(Egan et al. 1996)
IL8 (+)	GAGTTATTGAGAGTGGGCC	
IL8 (-)	AGCAGTCTAGGGTTGGAAG	(Legastelois et al. 1994)
IL10 (+)	TGTTGCCTGGTCTTCCTG	
IL10 (-)	TCTCTTCACCTGCTCCAC	(Gohin et al. 1997)
TNFα (+)	GAGCACAGAAAGCATGATCC	
TNFα (-)	AAAGTAGACCTGCCCAGACT	(Gohin et al. 1997)
ATPase (+)	GCTGACTTGGTCATCTGC	
ATPase (-)	CAGGTAGGTTTGAGGGGATAC	(Woodall, Maclaren, & Watt 1997)

Data analysis

Fluorescence intensity at each cycle was recorded by the proprietary Opticon Monitor (MJ Research; Reno, USA) software as a graph of intensity vs. cycle number, a representative example is shown in figure 2.2. The method used to extrapolate quantitative information from this data was as described by Higuchi et al (Higuchi et al. 1992;Higuchi et al. 1993). Briefly, PCR product from samples with more abundant template will start to exponentially accumulate following fewer reaction cycles, and the fluorescence intensity of the intercalated SYBR green dye rises proportionally with it (Lekanne Deprez et al. 2002). In order to analyse the quantity of starting template of each target gene, the earliest point at which it detectably enters the exponential amplification phase is determined. Subsequently this point is related to the equivalent point for the reaction product from one or more housekeeping gene (s) from the same experimental sample.

The suitability of ATPase as a housekeeping gene was assessed in short optimisation test prior to analysis of the experimental samples. Briefly, primers corresponding to three potential housekeeping genes – ATPase, GAPDH and β-actin were used for PCR fragment amplification. These were inserted into TOPO plasmids which were transformed into *E. coli*, which was grown to confluent culture. Colonies with verified plasmid inserts were selected and the plasmids purified, quantified and serially diluted. The serial dilutions of plasmids were used as the templates in real-time PCR reactions as previously described, and a standard curve derived for each gene. Bronchial epithelial brushing samples were then taken from experimental animals before and after LPS treatment, the RNA was purified and reverse transcribed to cDNA. These samples were then used as templates for each housekeeping gene and the results compared to the standard curves acquired previously. The results were

analysed by comparing the expression of each gene before and after LPS administration. The post-LPS sample results were divided by the baseline sample results to find a fold-change for each primer and the results compared using a student t-test to assess the significance of the results.

Both GAPDH and β-actin showed significant up-regulation (GAPDH 1.9 fold up regulated, p=0.001; β-actin 2.6 fold upregulated, p=0.003) following LPS exposure in the lung, and were therefore considered unsuitable for use as consistent housekeeping genes in this experiment. ATPase showed no significant change between samples (0.9 fold change, p=0.897), and was therefore considered to be suitable for use as an internal reference control for relative real-time PCR analysis. These results are concurrent with published data showing alterations in expression of both β-actin and GAPDH in lung disease (Glare et al. 2002).

In order to determine the point at which fluorescence rose above baseline and entered the exponential (quantitative) phase, the intensity background was subtracted from the overall totals at each point. Baseline threshold was set as two standard deviations above the minimum fluorescence over the total cycle range. The point at which fluorescence intensity crossed the threshold ($C_{(t)}$ value) was calculated, and this value was exported to a Microsoft Excel (Microsoft Corporation; Seattle, USA) worksheet for further analysis. The melting curve for each cytokine was analysed in order to ensure that fluorescence readings were due to genuine products of the appropriate size as opposed to non-specific amplification or primer-dimer formation. Any samples that did not conform to the standard shape of the melting curve were excluded from further analysis. An example of the melting curve graph obtained is given in figure 2.3.

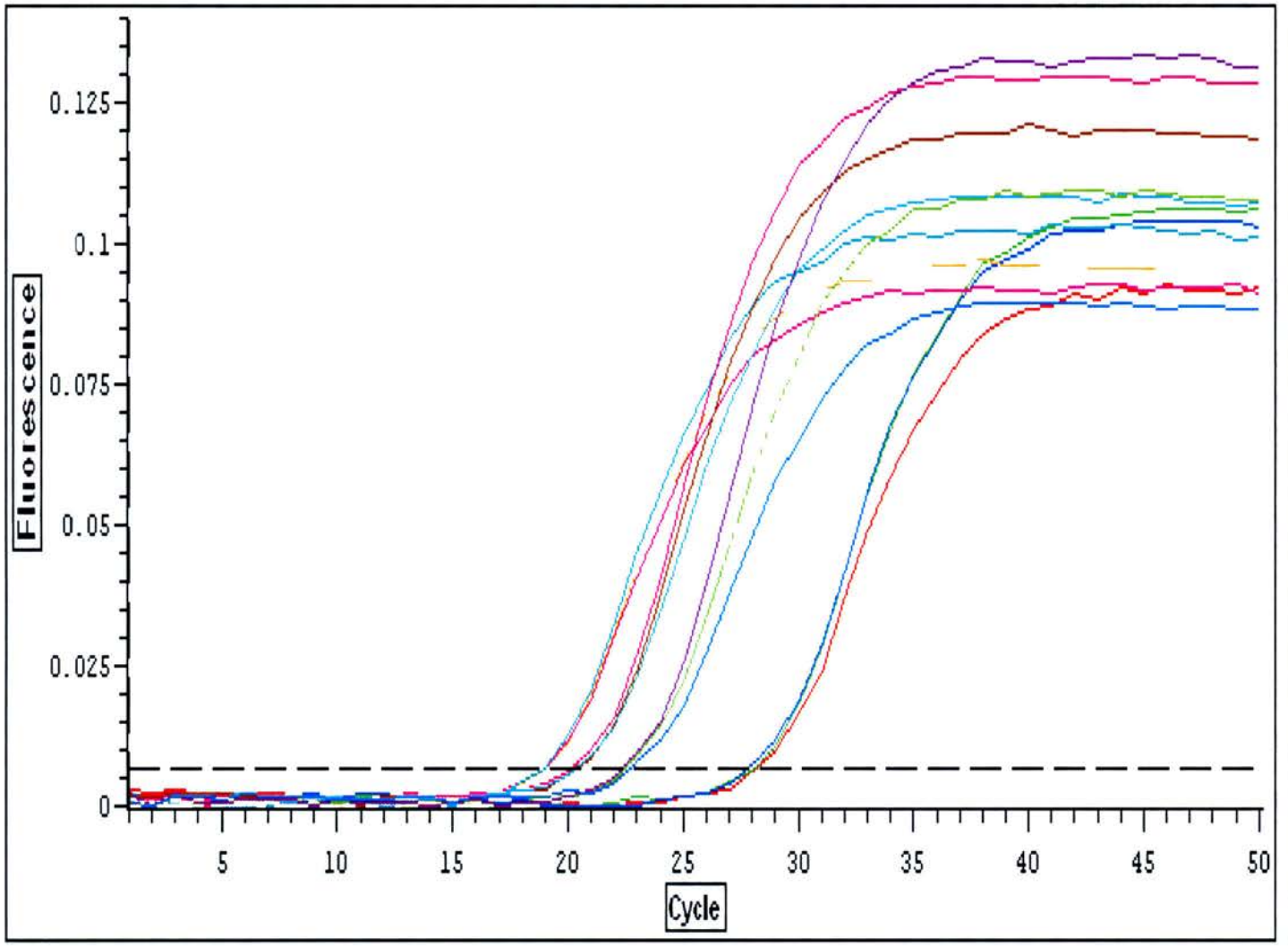


Figure 2.2: Representative example of a real-time PCR fluorescence intensity graph produced by the Opticon Monitor real-time PCR software. The x axis shows the cycle number at which fluorescence intensity is detected. Fluorescence intensity is proportional to the quantity of amplified PCR product. Each coloured line represents the fluorescence reading for an individual sample. Samples are loaded in triplicate. The point where each coloured line crosses the dashed threshold line is recorded as a $C_{(T)}$ value. This value is the point where amplification becomes exponential and thus the $C_{(T)}$ value is inversely proportional to the quantity of starting template.

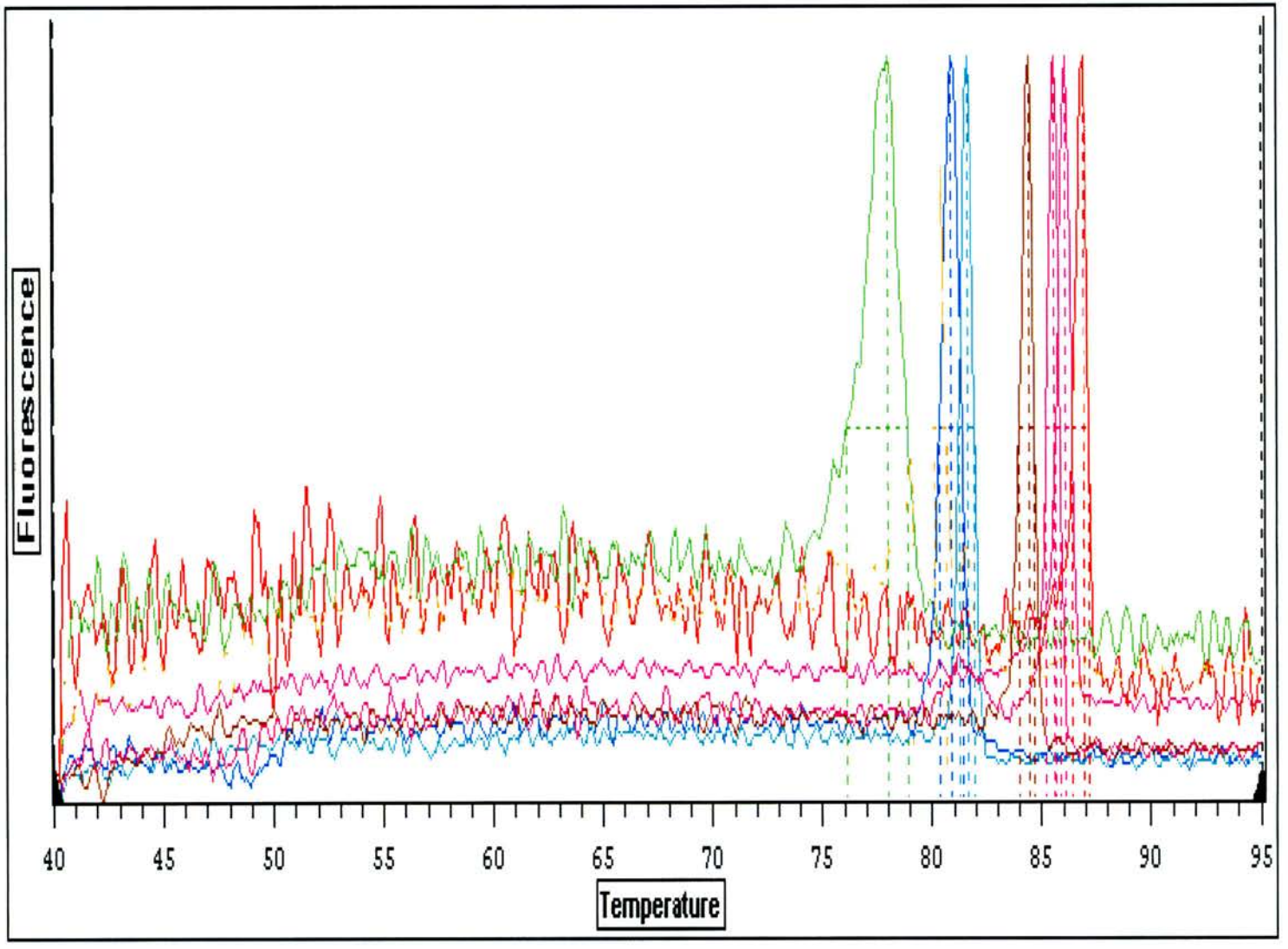


Figure 2.3: Example of a real-time PCR melting curve graph as produced by the Opticon Monitor software. Each coloured line represents a PCR sample. The integral of fluorescence intensity (y axis) is measured as temperature increases (x axis). At the melting temperature of any stable DNA product, a sharp peak is detected. Each peak represents a DNA fragment. A sharp, narrow peak represents a more stable and uniform DNA fragment. Higher peak temperatures represent longer DNA stretches. This technique is used to evaluate the quality of the PCR product within each sample replacing the gel in standard PCR.

The triplicate $C_{(t)}$ values for each sample were averaged to give an individual $C_{(t)}$ value for each sample. Each cytokine value was normalised to the corresponding ATPase value in order to give a relative expression value (R) using the formula:

$R = 2 \wedge (\text{ATPase C (t) value}) / 2 \wedge (\text{cytokine C (t) value})$ (Pfaffl, Horgan, & Dempfle 2002)

Outliers were identified using the Grubb outlier identification rule, and the remaining relative expression values were compared using a Wilcoxon signed rank test to identify significant changes in expression between samples at 0 hours and at 6 hours after LPS challenge. Significance was taken as $p \leq 0.05$. Fold changes in the expression level of each cytokine were calculated by dividing the 6 hour post-LPS value by the 0 hour baseline value.

Microarray processing

RNA amplification

The MessageAmp aRNA Kit (Ambion; Huntingdon, UK) is based on the RNA amplification protocol developed in the laboratory of Dr James Eberwine (Eberwine 1996). The procedure consists of reverse transcription with an oligo-dT primer bearing a T7 promoter and *in vitro* transcription of the resulting DNA with T7 polymerase to generate hundreds to thousands of antisense RNA copies of each mRNA in a sample. The antisense RNA is referred to as aRNA and the amplification method is referred to as the aRNA amplification procedure.

Prior to amplification 11.2 ml of ACS grade 100% ethanol was added to the cDNA wash and mixed well and 20 ml of ACS grade 100% ethanol was added to the bottle of aRNA wash and mixed well. Between 100 – 1000 ng of total RNA was placed into a sterile, RNase free 0.2ml PCR tube. The volume of this sample was 11 µl or below. To each PCR tube containing total RNA 1 µl Oligo-dT Primer was added along with

nuclease free water to give a final volume of 12ul. The mixture was incubated at 70°C for 10 minutes. The following were then added to each reaction: 10x First Strand Buffer (2 µl), Ribonuclease Inhibitor (1 µl), dNTP mix (4 µl). The samples were mixed by pipetting, centrifuged to collect the sample and placed on ice prior to incubation at 42°C with 1 µl of Reverse Transcriptase for 2 hours. Samples were centrifuged at <5000 rpm for 5 seconds to collect the reaction in the bottom of the tube and placed on ice. Second strand cDNA synthesis reagents were then added in the following order: nuclease free water (63 µl), 10x second strand buffer (10 µl), dNTP mix (4 µl), DNA Polymerase (2 µl), RNase H (1 µl). The reaction was pipetted to mix and incubated at 16°C for 2 hours.

cDNA was transferred to a clean, sterile, RNase free tube and 250 µl of cDNA. Binding Buffer added to each cDNA sample the sample was mixed thoroughly by repeated pipetting. The reaction was filtered through an equilibrated filter cartridge in a centrifuge at 10,000 g for 1 minute. 650 µl of cDNA Wash Buffer was added to each filter cartridge and the cartridge centrifuged for 1 minute at 10000 g. The filter cartridge was then centrifuged for a further minute at 10000 g to remove trace amounts of ethanol. cDNA was eluted by applying 10 µl nuclease-free water at 50°C, incubated at room temperature for 2 minutes, and then the cartridge was centrifuged at 10000 g for 90 seconds, this step was performed twice, and the purified cDNA stored at –20°C.

16 µl cDNA solution was transferred to a 0.2 ml sterile, RNase free PCR tube. To the 16 µl of double stranded cDNA, 4 µl of each of the following components were added in order, at room temperature (final volume = 40 µl). T7 ATP Solution (75 mM), T7 CTP Solution (75 mM), T7 GTP Solution (75 mM), T7 UTP Solution (75 mM), T7 10x Reaction Buffer and T7 Enzyme Mix. The reaction was incubated for 24 hours at

37°C. After the incubation 2 µl DNaseI was added to the reaction and the reaction incubated for 30 minutes at 37°C. 60 µl of elution solution was added to each sample and mixed thoroughly by repeated pipetting. Each sample was transferred to a fresh nuclease free 1.5 ml microfuge tube. 350 µl of aRNA Binding Buffer was added to each aRNA sample and mixed. 250 µl of ACS grade 100% ethanol was then added to each aRNA sample and mixed. Each aRNA/Binding Buffer/ethanol mix was then placed onto the center of an equilibrated filter cartridge and centrifuged for 1 minute at 10000 g. 650 µl of aRNA Wash Buffer was then applied to each filter cartridge and the cartridge centrifuged for 1 minute at 10000 g. The filter Cartridge was transferred to a fresh aRNA collection tube and 50 µl of Nuclease-free water at 50°C added. The solution was incubated at room temperature for 2 minutes and then centrifuged for 1.5 minutes at 10000 g. Elution was repeated with a second 50 µl of preheated Nuclease-free water. aRNA was stored at -80°C.

cDNA labelling

1.2 µg aRNA was diluted in 20 µl MilliQ water (Millipore; Livingstone, UK) in an RNase free microfuge tube. This was dried down in a SpeedVac centrifuge until the liquid had been removed from the tube. RNA was resuspended in 5 µl coupling buffer from the Stratagene Fairplay kit (Stratagene; Amsterdam, The Netherlands). The reaction was vortexed and spun briefly to collect the contents. Following incubation at 37°C for 15 minutes the tubes were vortexed and centrifuged briefly to return the contents. RNA was reverse transcribed as described above, with the inclusion of amino-allyl linked dCTP in the dNTP mix. 5 µl of Cy dye coupled to amino-allyl groups (Amersham plc.; Buckinghamshire, UK) was added to the cDNA and incubated for one hour at room temperature in the dark. This process linked the allyl groups and resulted in labelled cDNA. Labelled cDNA was purified using the DyeEx

2.0 spin kit (Qiagen; Sussex, UK) according to manufacturer's instructions. Briefly, the tubes were prepared by adding 300 µl MilliQ water (Millipore; Livingstone, UK) to the resin bed to resuspend the resin. The tubes were centrifuged at 3000 rpm for one minute in an Eppendorf microcentrifuge 5415D (Eppendorf UK Ltd; Cambridge, UK) before adding a further 300 µl MilliQ water and centrifuging the tubes at 3000 rpm for 3 minutes. Sample was added to the top of the suspended resin bed of the spin column, and the tubes centrifuged at 3000 rpm as before for 3 minutes. The eluate was then stored and used in the microarray hybridisation reactions.

Hybridisation

Cy labelled cDNA target was hybridized to DNA probes immobilized on a glass slide, using the Genomic Solutions GeneTAC automated hybridisation station (Genomic Solutions; Huntingdon, UK) according to manufacturer's instructions. This procedure was carried out in a clean room. Briefly, each array slide was placed within a GeneTAC cassette and heated to 75°C. The appropriate labeled cDNA solutions were then added to the cassette along with ULTRAHyb hybridization buffer (Ambion; Huntingdon, UK). The solutions were then agitated, hybridized and washed in a strictly controlled manner as part of the operation of the hybridization station, utilising proprietary hybridization solutions (Genomic Solutions; Huntingdon, UK). Once this process was complete the slides were immersed in low stringency buffer (Genomic Solutions; Huntingdon, UK) for approximately 1 minute followed by isopropanol for 1 minute. The slides were then dried by centrifuging (Beckman GS-3) at 1200 rpm for six minutes in preparation for scanning.

Scanning

Hybridised arrays were scanned using a ScanArray 5000XL (Perkin Elmer; Weybridge, UK) confocal laser microarray scanner according to the manufacturer's protocol. Arrays were scanned at 10 μm resolution for analysis. The laser intensity varied from 73-78% for the Cy3 and 75 and 78% for the Cy5 dye as appropriate for the intensity of fluorescence on each slide. Array scans for each dye were saved as .TIFF extension image files and stored for data acquisition.

Data acquisition

Data was acquired from the fluorescence intensity scan images using the BlueFuse data capture software package (BlueGnome; Cambridge, UK). BlueFuse measures signal intensity from each array spot present in the scanned image and corresponds each spot to its relevant data (name, position etc) using a pre-prepared procedure file designed around the .GAL file structure. The procedure file informs the program as to the layout of the printed slide and the relevant processing instructions. The acquired data is exported to an Excel spreadsheet (Microsoft Corporation; Seattle, USA) for analysis.

Data analysis

Data were analysed using the GeneSpring analysis software package (Silicon Genetics; Redwood, USA). Data were imported from excel files containing raw fluorescence intensity, background readings and array composition data. The software package transformed these raw intensities into relative values, and adjusted values according to background signal fluorescence, spot conformation and dye type. The transformed data were then analysed for dye-swap variance and normalized to the global mean of data as standard. Normalized data were plotted as scatter plots, and

analysed for significant changes using the segmental locations as replicates in a standard student t-test. The Benjamini and Hochberg False Discovery Rate method (Benjamini 1995) was used as a multiple testing correction where appropriate.

Chapter 3 Direct, remote and systemic effects associated with local lung instillation of LPS in sheep

Aims and objectives

- To define the nature of cellular events in the bronchoalveolar space following local lung instillation of lipopolysaccharide in sheep.
- To determine whether such local events are associated with concomitant systemic or 'lung-wide' changes.

Introduction

Acute local gram-negative sepsis is recognised as a predisposing factor in the development of acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) (Pelosi et al. 2003). Direct airway instillation of bacterial lipopolysaccharide (LPS) is a well-recognised rodent model of acute lung inflammation and ARDS (Holub & Lawrence 2003). LPS-induced airway inflammation is associated with a pronounced neutrophil (PMN) infiltrate (Wesselius et al. 1997), with attendant release of pro-inflammatory cytokines, proteases and free radicals leading to acute lung injury. This local inflammatory response is preceded by a systemic circulating neutrophilia (O'Grady et al. 2001), and the release of chemotactic factors at the site of stimulus resulting in the localisation of the circulating PMNs (Toews 2001). As only a proportion of individuals go on to develop ALI and ARDS it is conceivable that this variable susceptibility reflects a difference in the manner in which the lung responds as a whole organ to local insult. Indeed, whether such local insult acts to increase or decrease the sensitivity of the lung to further challenge is unclear, with conflicting reports in the literature (Miyazaki et al. 1999; Yamada et al. 2000).

Previous rodent model studies examining local and remote lung responses to acute bacterial sepsis reveal important limitations in study design. In a study by Terashima et al direct instillation of bacteria to one lung in order to study the contra-lateral response resulted in almost 20% of the instilled bacteria spilling over into the 'naïve' lung (Terashima et al. 1996). Further limitations relate to the close spatial relationship of individual lung lobes in rodent models with greater potential for crossover of inflammatory cells and mediators into contra lateral lungs and lung segments as a consequence of breakdown of compartmental barriers. This clearly confounds attempts to differentiate local and remote lung responses (Terashima et al. 1996). Large animals are less limiting in this respect and as such are a preferable model for segmental lung studies (Ferrari et al. 2003).

The sheep offers particular advantage as a model to study such responses. Sheep are tolerant of routine procedures such as blood sampling and do not constitute a particular anaesthetic risk (Borrie & Mitchell 1960). With specific respect to the respiratory system, sheep lungs are anatomically closer to those of man than are dog, cat or monkey lungs (McLaughlin et al. 1994) and when compared to dogs, sheep appear to be better suited as models for human gas exchange (Werlen, Py, & Haab 1984).

Previous studies have also demonstrate that the airway epithelium in sheep is highly analogous to human airway epithelium (Mariassy et al. 1988a; Mariassy et al. 1988b). Previous lung function analyses in the sheep include analysis of airway responses (Wagner & Mitzner 1990), lung inflammatory responses (Wheeler, Hardie, & Bernard 1992; Wheeler, Hardie, & Bernard 1990), lung function during anaesthesia , gas exchange of the lung (McNeil et al. 1991; McNeil et al. 1989) and physiological responses to exercise (Mundie et al. 1991). Sheep also benefit from ease of handling

and availability. Ovine immune and inflammatory responses appear analogous to those seen in human subjects responding to acute bacterial infection in the lung (Redl et al. 1993). The work detailed in this chapter characterises at a cellular level the direct, remote and systemic response to endobronchial instillation of LPS.

Results

BAL

Total cell counts

The total number of cells present in BALF (cells x 10^6/ml) was significantly ($p=0.001$, $n=12$) up-regulated six hours post-LPS challenge in the direct segment (0h median count: 2.04 x10^6 cells/ml [range: 0.69-6.94] vs. 6h median count: 9.28 x10^6 cells/ml [range: 2.37-23.86]). In BALF taken from the remote segment there was no significant change ($p=0.078$, $n=12$) in the total number of cells. Figure 3.1 shows, for each animal, the change in total cell numbers in BALF (cells x 10^6/ml) in the directly challenged and remote segments prior to treatment (0h), and at 6 hours after LPS challenge (6h).

Figure 3.1: Total cell counts

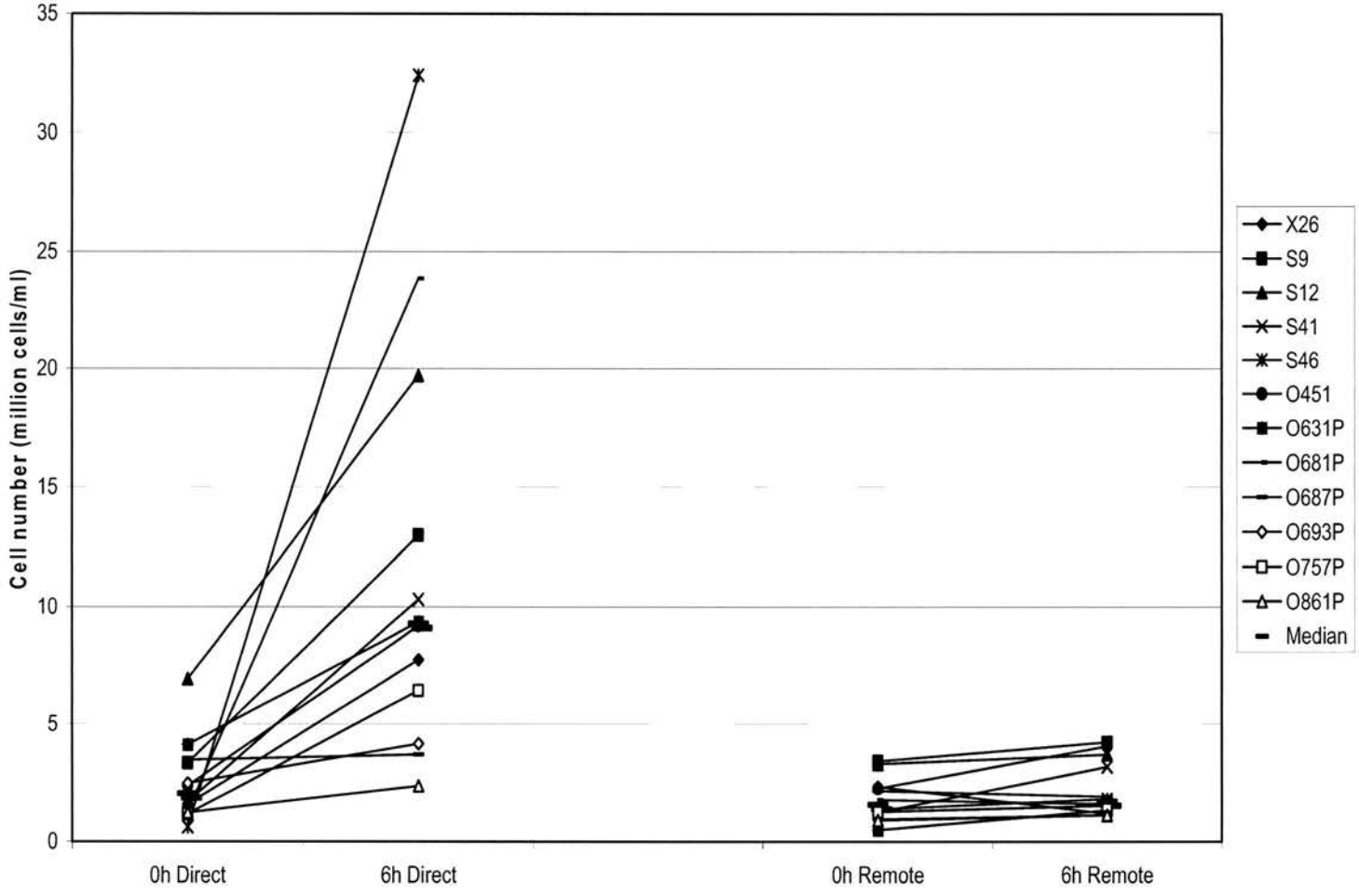


Figure 3.1: shows, for each animal, the change in total cell numbers (x 10^6 cells) in BALF in the directly challenged and remote segments prior to treatment, and at 6 hours after LPS challenge. Total cell count was significantly ($p=0.001$) increased six hours post-LPS challenge in the direct segment. Cells taken from the remote segment were not significantly changed ($p=0.078$) following contra lateral challenge.

Differential cell counts

Absolute differential cell counts in BALF (cells x 10^6/ml) derived from challenged (Direct) and contra-lateral (Remote) lung segments prior to treatment (0h) and 6 hours after LPS challenge (6h) are shown in table 3.1.

The total number of neutrophils present in BALF was significantly (p=0.001, n=12) increased six hours post-LPS challenge in BALF taken from the direct segment (0h median count: 0.03 x10^6 cells/ml [range: 0.002-0.76] vs. 6h median count: 5.89 x10^6 cells/ml [range: 0.23-18.08]). This represented the major part of the increase in the total cell numbers. Figure 3.2 shows, for each animal, the change in PMN numbers in BALF in the directly challenged and remote segments prior to treatment (0h), and at 6 hours after LPS challenge (6h). In BALF taken from the remote segment there was no significant change (p=0.784, n=12) in the number of neutrophils. No significant change in the total numbers of any other cell type in any segment resulted 6 hours following LPS challenge.

Systemic monitoring

Haematological analysis

The total number of neutrophils present in blood was significantly (p=0.002, n=12) increased six hours post-LPS challenge (0h median count: 0.88x10^6 cells/ml [range: 0-3.44] vs. 6h median count: 3.75 x10^6 cells/ml [range: 0.6-8.05]). The total number of lymphocytes present in blood was significantly (p=0.008, n=12) decreased six hours post-LPS challenge (0h median count: 4.8 x10^6 cells/ml [range: 1.59-11.13] vs. 6h median count: 2.44 x10^6 cells/ml [range: 1.15-5.48]). There were no significant changes in either total cell counts in blood or in any other specific cell types in blood

Table 3.1: Median total cell counts (x 10^6 cells) in bronchoalveolar lavage samples taken at baseline (0h) and 6 hours post-LPS challenge (6h). Segments used were labelled 'direct' for the segment into which LPS was instilled, and 'remote' for the contra-lateral segment sampled. Macrophage, lymphocyte and eosinophil numbers were not significantly altered in any segments following local LPS challenge. Neutrophil numbers were significantly higher in the directly challenged segment 6 hours post-LPS challenge than at baseline (p = 0.001).*

	0h Direct	6h Direct	0h Remote	6h Remote
Macrophage	1.50	1.81	1.38	1.17
Neutrophil	0.03	7.68*	0.04	0.03
Lymphocyte	0.05	0.16	0.04	0.03
Eosinophil	0	0	0	0

Figure 3.2: PMN cell counts

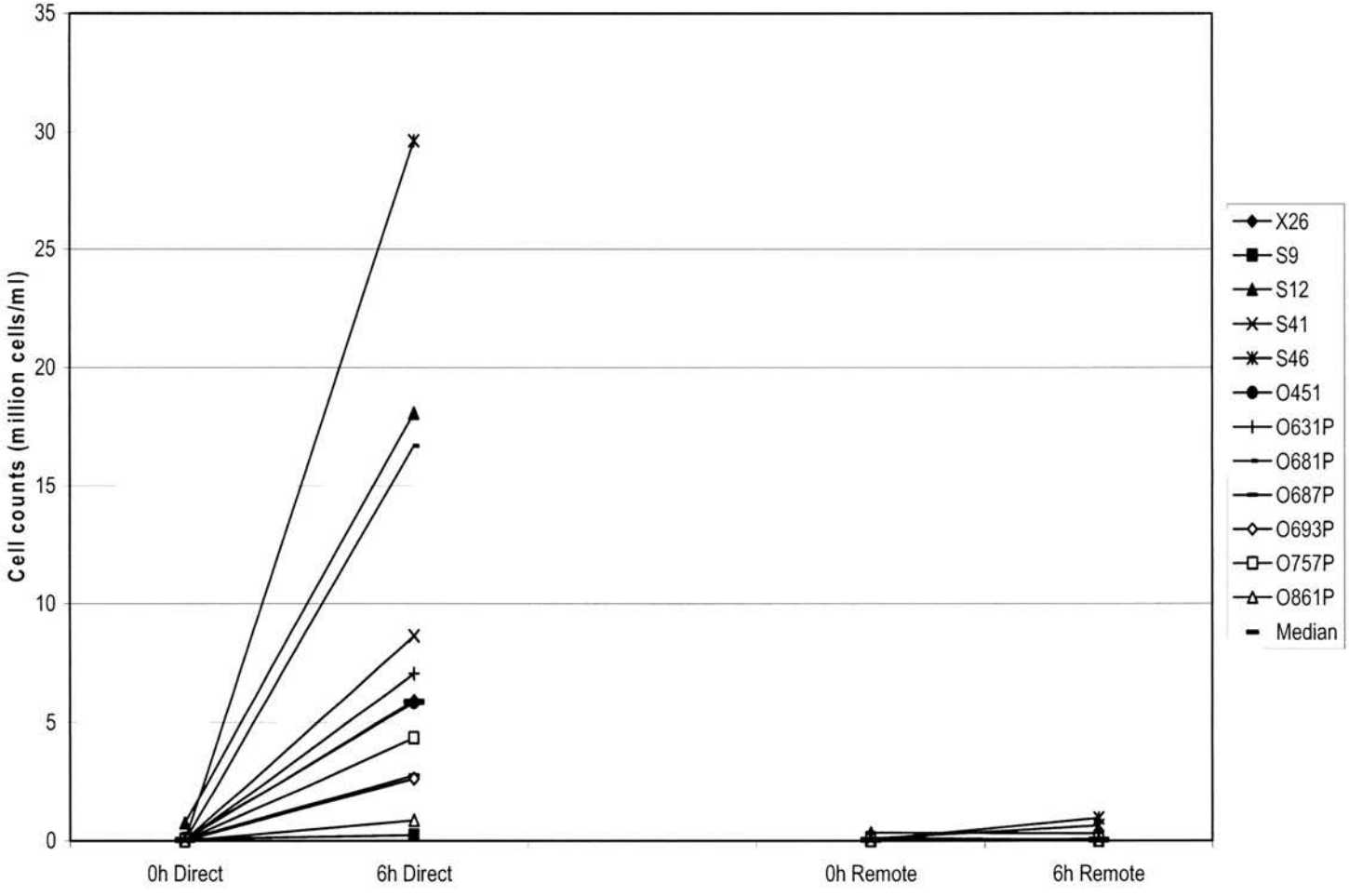


Figure 3.2: shows, for each animal, the change in PMN numbers (x 10^6 cells) in BALF in the directly challenged and remote segments prior to treatment, and at 6 hours after LPS challenge. Total neutrophil numbers were significantly increased (p=0.001) six hours post-LPS challenge in BALF taken from the direct segment. This represented the major part of the increase in total cell numbers. In BALF taken from the remote segment there was no significant change (p=0.784) in the number of neutrophils.

6 hours following LPS challenge. The systemic haematological effects of local lung administration of lipopolysaccharide are depicted in table 1 (Appendix I).

Rectal Temperature

A slight (+0.8 °C) but significant ($p<0.005$) pyrexia developed as a consequence of the procedure (Table 2; Appendix I). This mild pyrexia was not associated with any clinical signs of systemic disease.

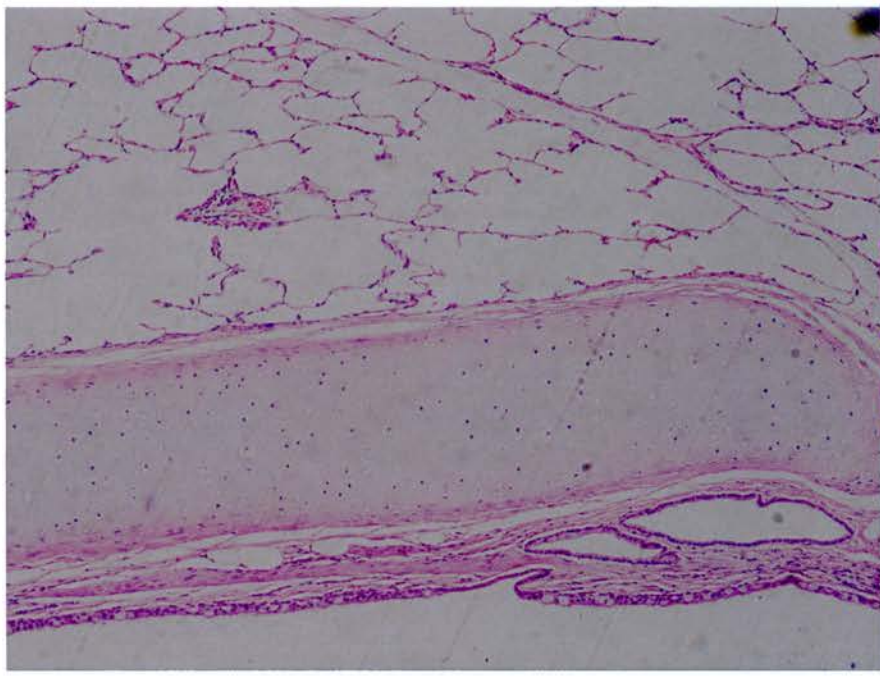
Histopathology

Figure 3.3 shows a section of lung from a healthy, untreated animal at x 40 (i) and x 400 (ii) magnification and represents the normal histology of the ovine lung. In sections from the directly challenged segments there was clearly defined pathological change. The predominant pathological change observed were a moderate to severe infiltration of neutrophils into the lumena of small (terminal) bronchioles and particularly the alveolar air spaces. In the most severely affected areas there were also varying degrees of intra-alveolar oedema and occasionally mild haemorrhage. These acute inflammatory changes are within discrete, although poorly defined foci, adjacent to unaffected areas; this may be due to uneven distribution of LPS within the lung segment.

Figure 3.4 is a digital image of a section taken from a directly challenged segment at x 40 (i) and x 400 (ii) magnification and is representative of these changes. These changes are consistent with acute, neutrophil mediated inflammation seen in clinically and experimentally inflamed sheep lungs (R. Blundell; personal communication, 2004). The histopathological features of sections derived from adjacent and remote segments were consistent with those seen in untreated control animals managed under identical conditions. These data, particularly the example from the adjacent section,

illustrate the highly localised and site-specific effects of local LPS deposition by bronchoscopy.

Figure 3.5 shows a representative section from a segment adjacent to the challenged segment at x 40 (i) and x 400 (ii) magnification and figure 3.6 shows a representative section from a segment contra lateral to the challenged segment at x 40 (i) and x 400 (ii) magnification. Neither section shows any detectable histological abnormalities.



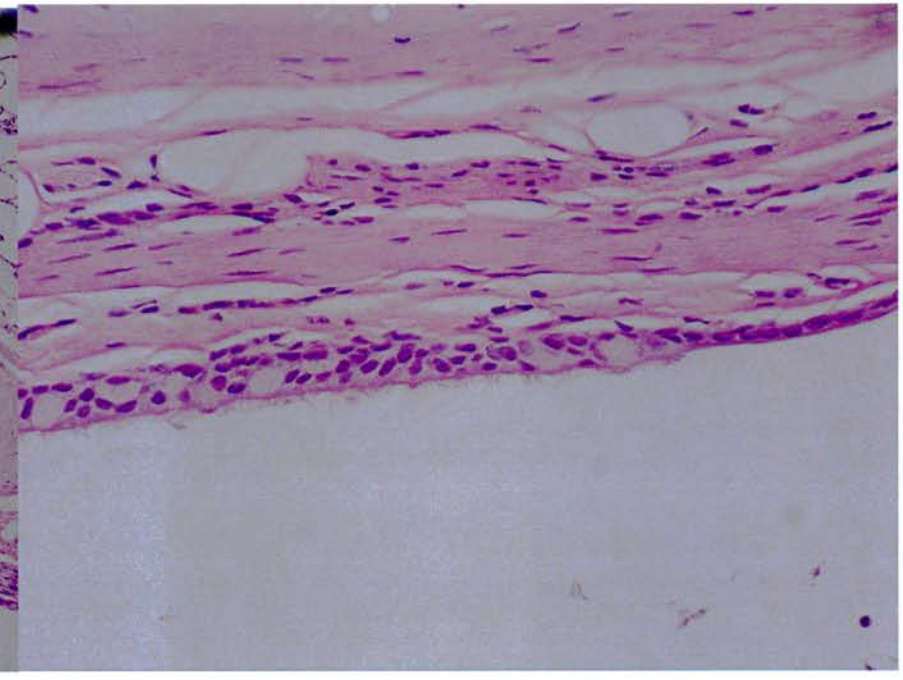


Figure 3.3.i: Digital image of a formalin-fixed, paraffin embedded tissue section taken from a naive segment (x 40 magnification). The tissue section shows morphology and histology consistent with healthy, un-inflamed lung. The alveolar spaces are clear of infiltrating immune cells, the airway wall is intact and the epithelial layer is undamaged. Resident cells are at normal levels and there is no evidence of haemorrhage or oedema. The section was assessed by a veterinary pathologist to confirm these observations.	*Figure 3.3.ii: Digital image of a formalin-fixed, paraffin embedded tissue section taken from a naive segment (x 400 magnification). The tissue section shows morphology and histology consistent with healthy, un-inflamed lung. The epithelium contains normal quantities of resident immune cells, the airway wall is intact and the epithelial layer is undamaged, with clear ciliated epithelial cells visible. There is no evidence of haemorrhage or deformation of airway cells. The section was assessed by a veterinary pathologist to confirm these observations.*

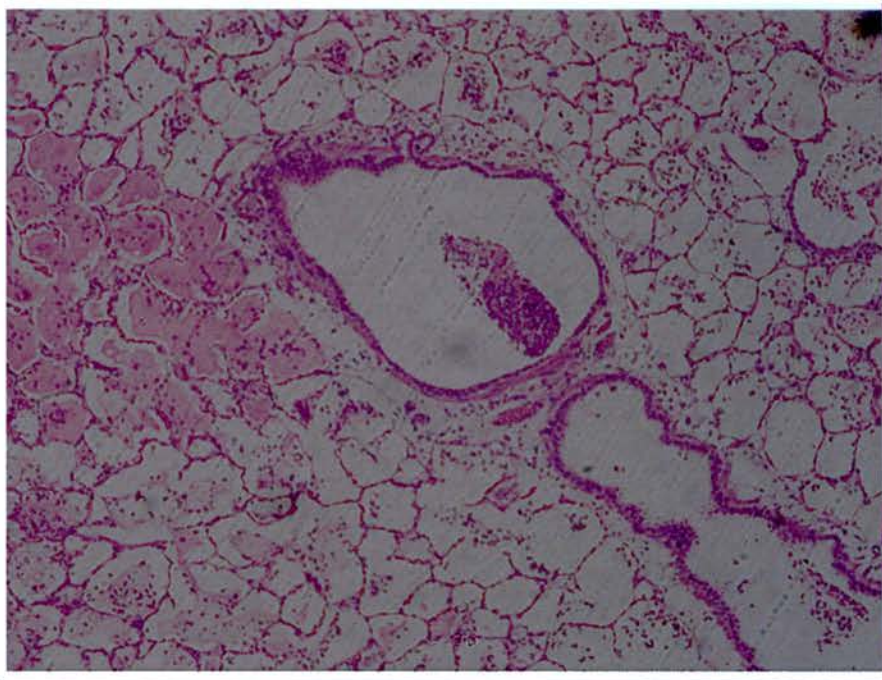


Figure 3.4.i: Digital image of a formalin-fixed, paraffin embedded tissue section taken from a lung segment directly challenged with LPS (x 40 magnification). The tissue section demonstrates significant pathological change. A moderate to severe infiltration of neutrophils into the lumina of small (terminal) bronchioles and particularly the alveolar air spaces is apparent. There is also intra-alveolar oedema and mild haemorrhage apparent. The section was assessed by a veterinary pathologist to confirm these observations.

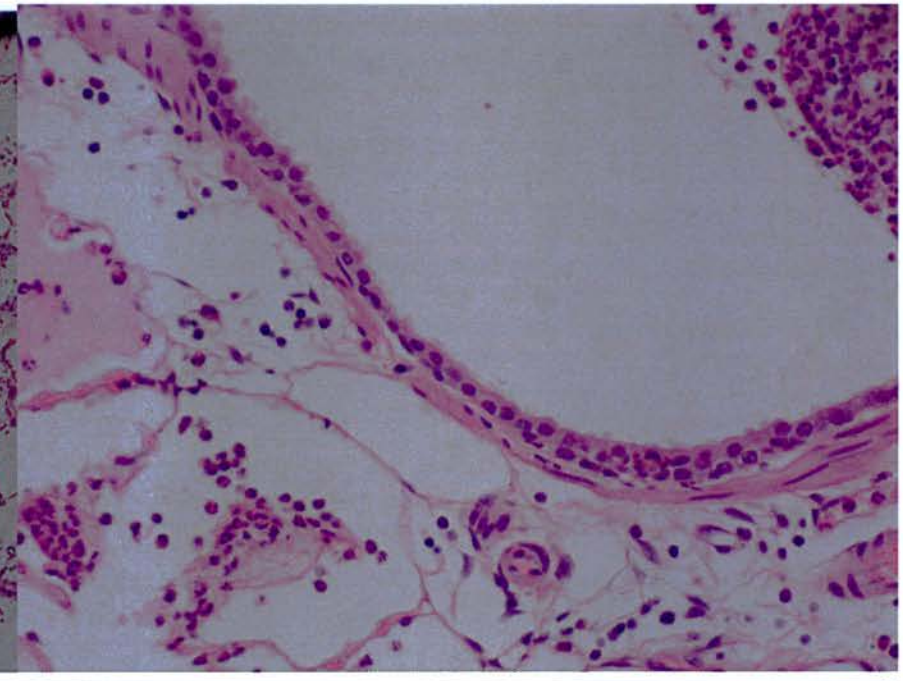


Figure 3.4.ii: Digital image of a formalin-fixed, paraffin embedded tissue section taken from a lung segment directly challenged with LPS (x 400 magnification). The tissue section demonstrates significant pathological change. The tissue contains increased numbers of immune cells although the airway wall and epithelial layer are intact, the ciliated epithelial cells show altered morphology. There are neutrophil aggregations visible in the airway and alveolar spaces. The section was assessed by a veterinary pathologist to confirm these observations.

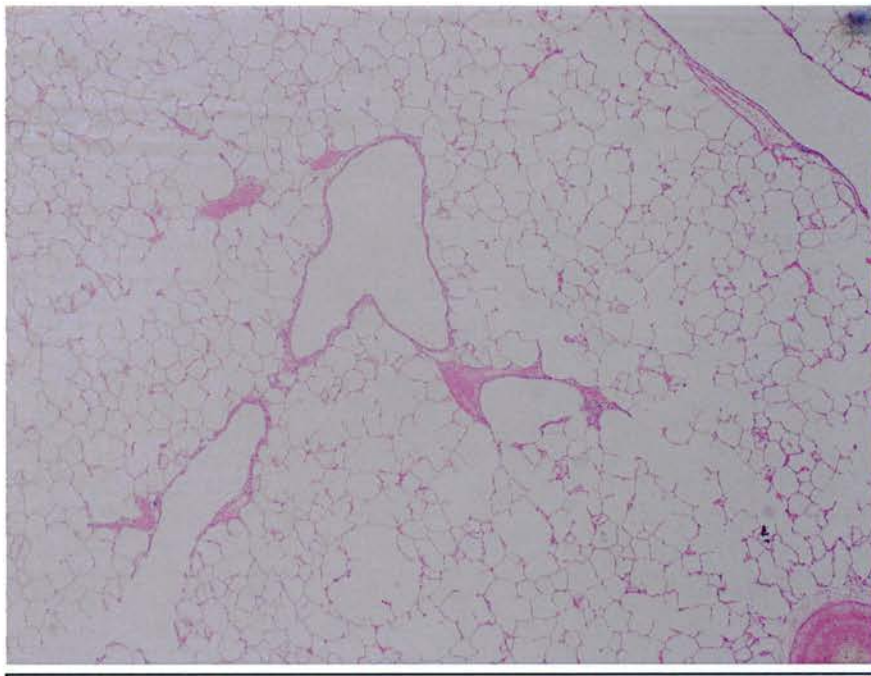


Figure 3.5.i: Digital image of a formalin-fixed, paraffin embedded tissue section taken from a lung segment adjacent to the LPS-challenged segment (x 40 magnification). The tissue section shows morphology and histology consistent with healthy, un-inflamed lung and similar to that of the naïve segment. This slide highlights the local nature of the inflammatory response to segmental LPS instillation. The section was assessed by a veterinary pathologist to confirm these observations.

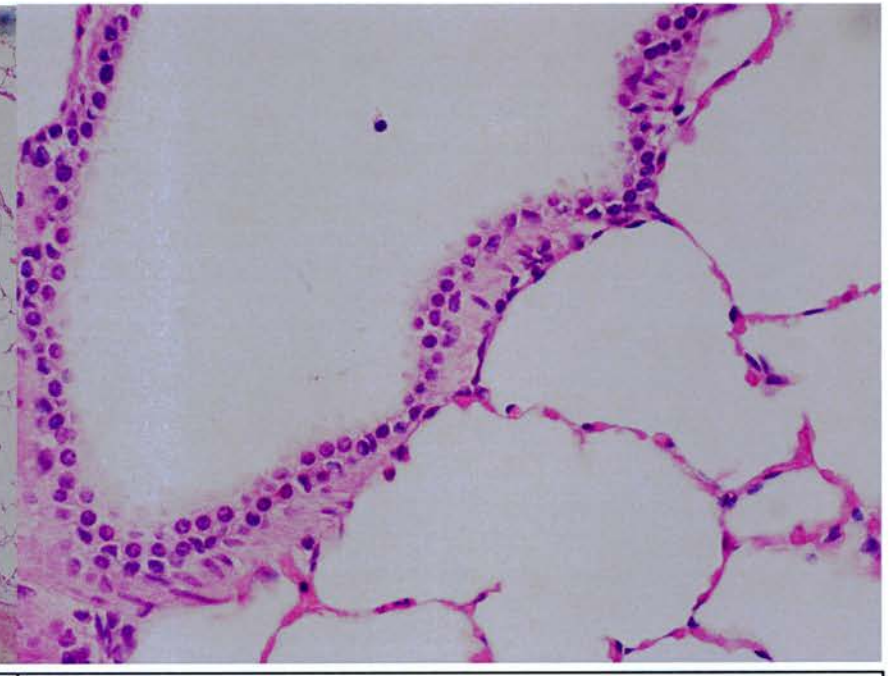


Figure 3.5.ii: Digital image of a formalin-fixed, paraffin embedded tissue section taken from a lung segment adjacent to the LPS-challenged segment (x 400 magnification). The tissue section shows morphology and histology consistent with that seen in the naïve segment. Resident cells are at normal levels and there is no evidence of haemorrhage or oedema. There does not appear to be any change in the neutrophil levels in the segment. The section was assessed by a veterinary pathologist to confirm these observations.

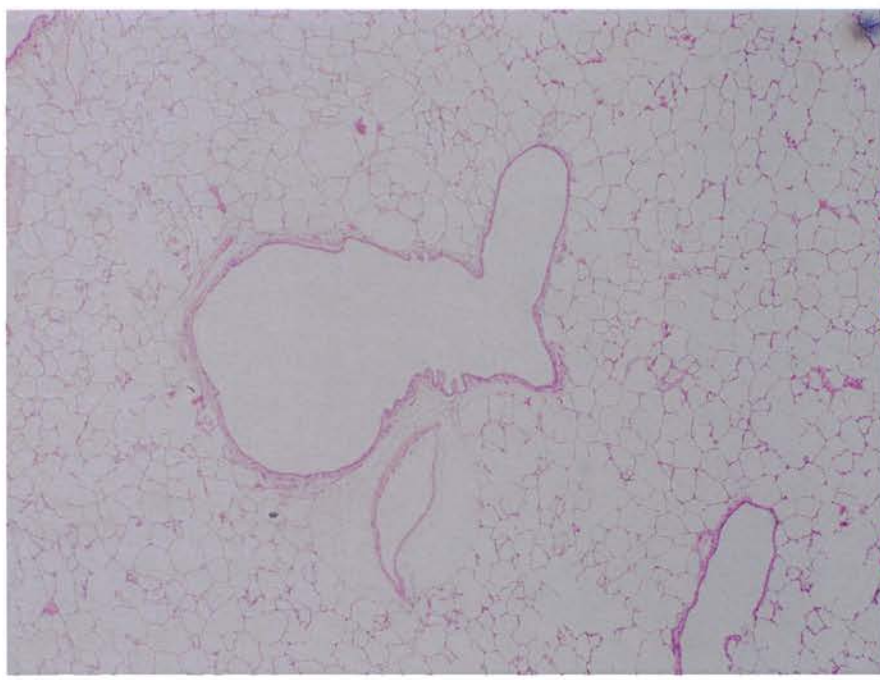


Figure 3.6.i: Digital image of a formalin-fixed, paraffin embedded tissue section taken from a lung segment contra lateral to the LPS-challenged segment (x 40 magnification). The tissue section shows histology consistent with healthy, un-inflamed lung and similar to that of the naïve and adjacent segments. This slide indicates the local nature of the inflammatory response to segmental LPS instillation and confirms the lack of gross change in the remote lung following local LPS challenge. The section was assessed by a veterinary pathologist to confirm these observations.

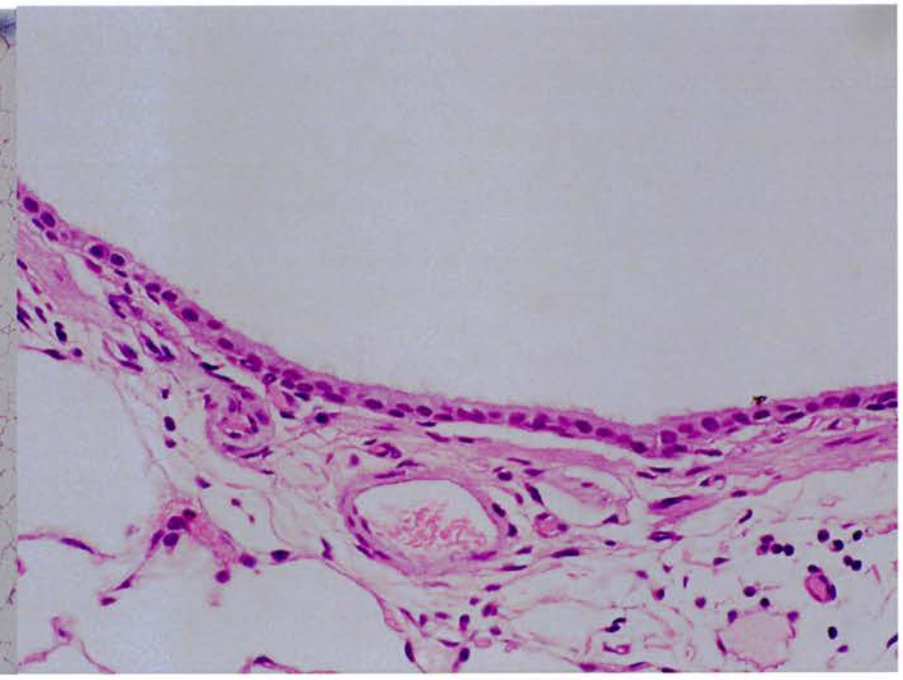


Figure 3.6.ii: Digital image of a formalin-fixed, paraffin embedded tissue section taken from a lung segment contra lateral to the LPS-challenged segment (x 400 magnification). The section shows histology consistent with healthy lungs. Resident cells are at normal levels and these observations are consistent with those from the naïve and adjacent segments. There is no change in the neutrophil content of the segment, confirming the lack of gross impact of local LPS challenge on the contra-lateral lung. The section was assessed by a veterinary pathologist to confirm these observations.

Discussion

Segmental approaches have been extensively utilised in the characterisation of the functional, cellular, and immune nature of the local lung response to antigen challenge in both experimental animals and humans. Published studies have included allergen challenge in dogs (Out et al. 2002; Bice & Muggenburg 1996; Bice, Jones, & Muggenburg 1993; Jones et al. 1993; Weissman et al. 1992), horses (Yoshikawa et al. 2003; Hoffman et al. 1993), pigs (Goodman et al. 1991) and human subjects (Sur et al. 1996; Shaver et al. 1995; Hunt et al. 2002). Sheep have been used in a similar manner to model a number of human lung diseases including emphysema (Susskind et al. 1985; Peterson et al. 1992; Janoff et al. 1983) and allergic lung disease (Collie et al. 2001).

This study indicates that local lung instillation of LPS in sheep elicits an inflammatory response at 6h after instillation. The inflammation remains confined to the challenged segment and there is no evidence of cellular change in contra lateral segments. The discrete and focal nature of the inflammatory response is supported by histopathological analysis of the tissues where contra lateral and indeed adjacent segments show no evidence of an inflammatory response. However, instillation was associated with systemic changes in the form of a pyrexia and blood neutrophilia. Overall the model appears to offer the facility to determine whether apparently discrete events at a local lung level can modify gene expression throughout the lung as a whole.

Bacterial sepsis involving the lung is associated with increased numbers of PMN in the circulation with a concomitant local increase in PMN airway infiltrates in the absence of involvement of the contra lateral lung (Monton & Torres 1998). Bacterial sepsis and associated inflammation has been shown to be a contributory factor in the

development of ARDS (Pelosi et al. 2003). The results of this study indicate that the response to endobronchial LPS instillation to local lung segments closely mirrors the accepted model of Gram negative bacterial inflammation (Moore & Standiford 1998). The response in the direct segment is as described for acute bacterial infection in both sheep (Brogden, Cutlip, & Lehmkuhl 1984) and humans (O'Grady et al. 2001). Cellular content of BALF from the directly challenge segment consisted of increased neutrophil numbers.

In contrast, the contra-lateral lung showed no change from baseline following LPS challenge. Brogden et al (1995) highlighted, in sheep and cattle, a localised, neutrophil-mediated inflammatory response to LPS instillation consistent with that seen in this study (Brogden, Ackermann, & Debey 1995). A study in human lungs by O'Grady et al (2001) in which local LPS deposition elicited an inflammation at the cellular and cytokine level showed considerable similarity with the results seen in this experiment(O'Grady et al. 2001). Haematological analysis indicated the presence of a systemic neutrophilia with a reduction in total lymphocyte numbers.

These data, consistent with the reactions seen in previously described models of lung endotoxaemia (Toews 2001; Xing et al. 1999), were not associated with any clinical evidence of disease. Histopathological analysis of the segments directly challenged with LPS showed damage consistent with neutrophil mediated damage associated with bacterial infection whilst the contra-lateral segment indicated no damage remote from the site of inflammation. The mild systemic pyrexia seen in this study was not of clinical significance and was consistent with sham procedures seen in previous studies (Collie; personal communication, 2004).

There are a number of possible mechanisms for the systemic production of neutrophils following exposure to endotoxin. It is conceivable that these cells are

produced in response to endotoxin reaching the systemic circulation across the airway barriers. Indeed tight junction perturbation may presumably occur prior to or in the absence of significant evidence of epithelial damage. A further possibility, supported by *in vitro* investigations (Ahmad et al. 2003; Fujii et al. 2001) is that the LPS initiates immune mediator production from resident cells lining the airspaces and/or the mobile cell population in the epithelial lining fluid and that raised local and/or systemic levels of these factors, which may include cytokines, chemokines or chemotactic factors, initiate the production of PMNs and facilitate the localisation of the PMNs to the site of inflammation.

The preferential influx of the PMNs to the inflamed lung may also be facilitated by the increased expression of adhesion molecules and localisation signals such as selectins and ICAM molecules as a direct result of LPS treatment (Kuo et al. 2000; Wright et al. 1991) as well as indirectly via the action of mediator cells such as alveolar macrophages etc (Blahnik et al. 2001). The exposure of PMNs to LPS, known as priming of the cells, has been shown to result in a reduction of the PMNs ability to deform (Lawrence et al. 1996;Sato et al. 1999), and hence they become more 'rigid' and less able to transfer across barriers from the circulation. Priming may help to explain the localised nature of the PMN response as the primed, less deformable cells do not readily cross barriers such as airway epithelium, and will thus be less likely to transfer without additional chemotactic signalling.

Once located in the lung airspaces the neutrophils will be preferentially retained due to the lack of deformability acquired through the priming of the cells by LPS exposure. In conclusion, available literature suggests that the combination of priming and adhesion molecule expression would contribute to the observed highly focal infiltration of PMNs in the lung following localised deposition of LPS.

An emerging hypothesis concerning the advancement of lung inflammation from a localised and relatively minor inflammatory response to the destructive and highly lethal ARDS syndrome concerns the balance between pro- and anti-inflammatory reactions in the lung following exposure to infectious agents.

The previously accepted paradigm of this evolution of inflammation held that the progression to ARDS was due to a loss of anti-inflammatory capacity in the lung and excessive pro-inflammatory reaction (Bone et al. 1992) and that this resulted in the inflammatory reaction overwhelming the organ and consequently causing tissue damage and organ failure leading to death in many cases (Bone, Sibbald, & Sprung 1992). This hypothesis was supported by evidence of therapeutic benefit following systemic delivery of the anti-inflammatory cytokine IL 10 in animal models of tissue infection and sepsis, but was confounded by research indicating that if the IL 10 was administered at the site of bacterial infection there was a delay in clearing bacteria and an increase in mortality.

These studies indicated that a systemic anti-inflammatory reaction was highly protective, however a local, tissue based, anti-inflammatory reaction resulted in greater susceptibility to adverse effects from infection and increased mortality following infection. Suggested mechanisms for this enhanced susceptibility include impairment of macrophage and monocyte recognition of, and response to, bacterial molecules (such as LPS), impaired neutrophil function and reduced levels of surfactant proteins in the airway space (Munford & Pugin 2001).

The macrophage has been established as a playing a crucial role in the resolution of bacterial infection and loss of function in macrophages has been shown to compromise the response to bacterial challenge to the lung. It has been clearly demonstrated that macrophage functions are severely reduced following direct

exposure to LPS, and also that this exposure consequently renders the macrophages less effective in terms of response to bacterial challenge (Dobrovolskaia & Vogel 2002; West & Heagy 2002). It has also been noted that macrophage function is impaired following exposure to IL 10, with a similarly compromised response to bacteria. An interesting additional observation was made by Dehoux who demonstrated that, following unilateral pneumonia involving localised inflammatory responses and no gross involvement of the contra lateral lung, macrophages isolated from the contra lateral lung segments were hyporeactive to LPS treatment (Dehoux et al. 1994).

The importance of the neutrophil in resolving bacterial lung infection is also well established (Aldridge 2002; Chollet-Martin et al. 1996), though it is clear that PMNs can also mediate considerable tissue damage, and have been implicated in the hyper responsive inflammatory effect seen in some lung diseases. Impairment of neutrophil function is known to exacerbate the negative effects of bacterial colonisation of the lung and loss of function in neutrophils correlates strongly with morbidity and mortality following endotoxin administration to the lung (Frevert, Warner, & Kobzik 1994; Rehm, Gross, & Pierce 1980; White et al. 1986).

Patients with sepsis have been shown to have impaired PMN function, showing reduced chemotaxis towards endogenous and exogenous factors *in vitro*. PMNs have also been shown to exhibit reduced migration to secondary sites of inflammation in septic patients (Ahmed et al. 1999). This impairment of function suggests that patients with sepsis are at increased risk of secondary infection due to loss of PMN antibacterial properties, as has also been reported in animal models of sepsis.

The phenotypic characteristics of the direct response to LPS at 6 hours post-challenge are consistent with activation of the innate acute inflammatory arm of the immune

system and prior literature suggests that such responses are likely to be accompanied by a characteristic profile of cytokine production from the resident immune and epithelial cells in the airways and alveoli. Such cytokines are likely to include TNFα, IL 1, IL 6, IL 8 and IFNγ (Toews 2001).

That no remote responses were detected, either at the level of the bronchoalveolar cellular profile or following histological examination, is consistent with previous studies (Monton & Torres 1998). The lack of organ-wide phenotypic change in this model supports the contention that local lung responses remain discrete. Whilst such an observation makes intuitive sense in that local restriction of an inflammatory focus would be desirable it does raise the question as to whether mechanisms exist at an organ level to limit progression, and indeed whether progression to fulminant inflammatory lung disease – as seen in ARDS - represents a failure of such mechanisms, and thus supports the hypothesis that lung failure is a consequence of disruption in the balance of pro- and anti-inflammatory responses. Resolution of this question would lead to a deeper understanding of the mechanisms of pathogenesis of sepsis in the lung and consequently widen the available options for therapeutic strategies or interventions in the treatment of lung infection.

Chapter 4 Direct, and remote cytokine expression associated with local lung instillation of LPS in sheep

Aims and objectives

- To validate the process of deriving quantitative information relating to endogenous gene expression from samples of bronchial epithelial cells obtained by bronchoscopic cytology brush *in vivo*.
- To characterise the molecular profile of cytokine gene expression in bronchial epithelial cells in response to direct lung challenge with LPS.
- To characterise the concomitant response in terms of cytokine gene expression in lung segments remote to the sites of direct challenge.

Introduction

Over the last 30 years flexible bronchoscopy has become established as a routine clinical diagnostic procedure (Mitchell et al. 1980a; Mitchell et al. 1980b). However it is only relatively recently that the potential of this technique to furnish specimens appropriate for molecular analysis has become apparent (Eissa & Erzurum 2001). The majority of recent studies have utilised biopsy material in this regard. However, although cytological brushing is a method that can be employed to harvest viable tracheobronchial epithelial cells for similar analyses (Kelsen et al. 1992), relatively few studies have capitalised on this technique in a clinical research context (Trapnell 1993; Becker et al. 1999; Penn et al. 1996; Riise et al. 1996; Bhathena et al. 2000; Joseph et al. 2001; Takizawa et al. 2000; Takizawa et al. 2001; Hackett et al. 2003). As such, the practical aspects in relation to sampling and processing for molecular analysis are not sufficiently well described or widely validated to be considered a

routine adoption in any experimental protocol. It is for this reason that the first objective is stated.

The second and third objectives, aimed specifically at the underlying hypothesis, are a natural progression from chapter 3. Primer pairs were synthesised in order to detect transcripts pertaining to the expression of IL 1β, IL 6, IL 8, TNFα and IL 10 in epithelial cells sampled before, and 6h after local lung instillation of LPS in the ovine model and to quantify this expression in relation to the housekeeping gene ATPase.

Results

RNA quality and quantity

Table 4.1 lists the quantity of RNA from each sample derived from the lung segments in this study. Figure 5.3.A is a representative example of the 'false gel' image acquired by the Agilent Bioanalyzer chip analysis platform. Full trace scans of all samples are shown in Appendix II. A number of the PCR data show numbers used in statistical tests less than 12. Samples were excluded from the data analyses due to PCR contamination or error established by analysis of melting curve data for each sample as detailed in the materials and methods chapter.

*Table 4.1: This table details the quantity of total RNA (μg) extracted from the brushed epithelial cells derived from each sheep lung sample. Samples labelled 'direct' were taken from the LPS-challenged segment, samples labelled 'remote' from the contra lateral lung segment at baseline (0 hours) and post-LPS challenge (6 hours). The quantification of the RNA was performed using the Agilent Bioanalyzer platform which calculates RNA concentration using fluorescence-based detection technology. The RNA was purified and divided into two. The first half of each sample was used for real-time quantitative RT-PCR analysis of cytokines. The second half was used for amplification and labelling prior to microarray hybridisation (see chapter 6). The RNA analysis was performed on the second half of each sample after the first half had been reverse-transcribed for the RT-PCR reactions. Due to a technical error the second half of 13 RNA samples were destroyed (marked as * in the table). Samples labelled ** were not amplified or used in the microarray analysis due to quantity and/or quality considerations.*

Sheep	Direct (0h)	Direct (6h)	Remote (0h)	Remote (6h)
O687P	199.5	362.5	251.5	308.5
O693P	4**	161.5**	420.5	208
O861P	0.5**	18.5**	190	108.5
O681P	262	56	177	217.5
O631P	276.5	11	144.5	264.5
O757P	180.5	70	147	165
451	*	3**	213.5	173
S12	*	*	*	*
S46	118.5	117.5	106.5	142.5
X26	*	*	81.5	34.5
S9	*	*	*	*
S41	*	*	106.5	142.5

Real-time PCR analysis of cytokine expression

The raw data obtained from the real-time PCR analysis are included in Appendix II.

Interleukin 1β

The relative expression of interleukin 1β mRNA as measured by qRT-PCR was significantly ($p=0.002$, $n=11$) up-regulated six hours post-LPS challenge in epithelial brushings taken from the direct segment (median fold difference relative to baseline: 248.9; range: 1.1-30829.5). In epithelial brushings taken from the remote segment there was no significant change ($p=0.919$, $n=10$) in the level of IL 1β expression. Figure 4.1 shows, for each animal, the change in relative expression of IL 1β in epithelial brushings in the directly challenged and remote segments both prior to (0h) and 6 hours after LPS challenge (6h).

Interleukin 6

The relative expression of interleukin 6 mRNA was significantly ($p=0.01$, $n=12$) up-regulated six hours post-LPS challenge in epithelial brushings taken from the direct segment (median fold difference relative to baseline: 4.6; range: 0.04-48.6). In epithelial brushings taken from the remote segment there was no significant change ($p=0.505$, $n=11$) in the level of IL 6 expression. Figure 4.2 shows, for each animal, the change in relative expression of IL 6 in epithelial brushings in the directly challenged and remote segments both prior to (0h) and 6 hours after LPS challenge (6h).

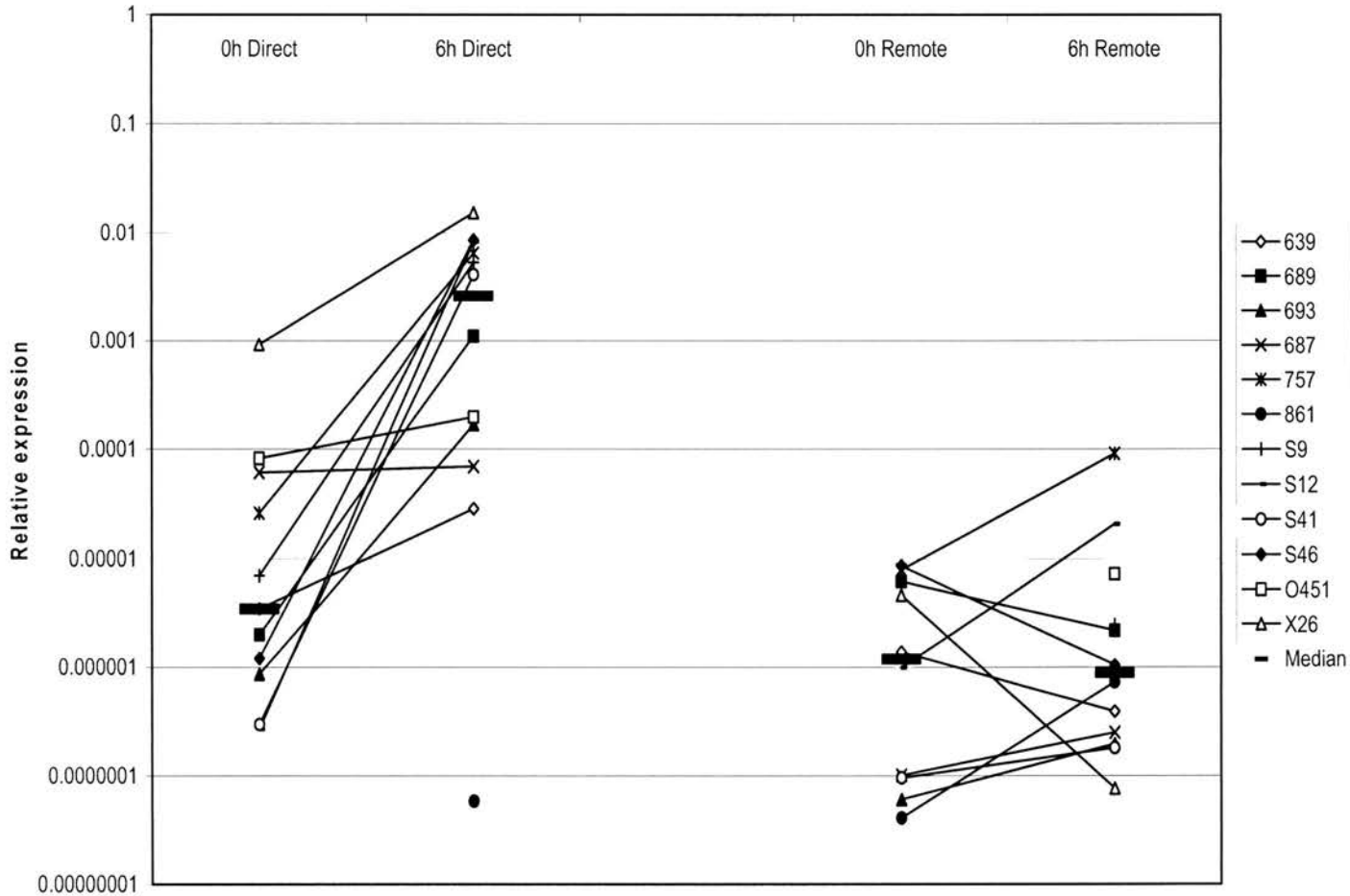


Figure 4.1: Expression level of interleukin (IL) 1β as assessed by real-time RT-PCR analysis of RNA from brushed epithelial cells in challenged (direct) and contra lateral (remote) segments at baseline (0 hours) and post-LPS (6 hours). Expression level relative to ATPase housekeeping gene expression is shown on the y-axis on a logarithmic scale. IL 1β was significantly (p=0.002) increased(mean 248.9 fold change) six hours post-LPS challenge in epithelial cells taken from the direct segment. In the epithelial cells taken from the remote segment there was no significant change (p=0.919) in the expression if IL 1β.

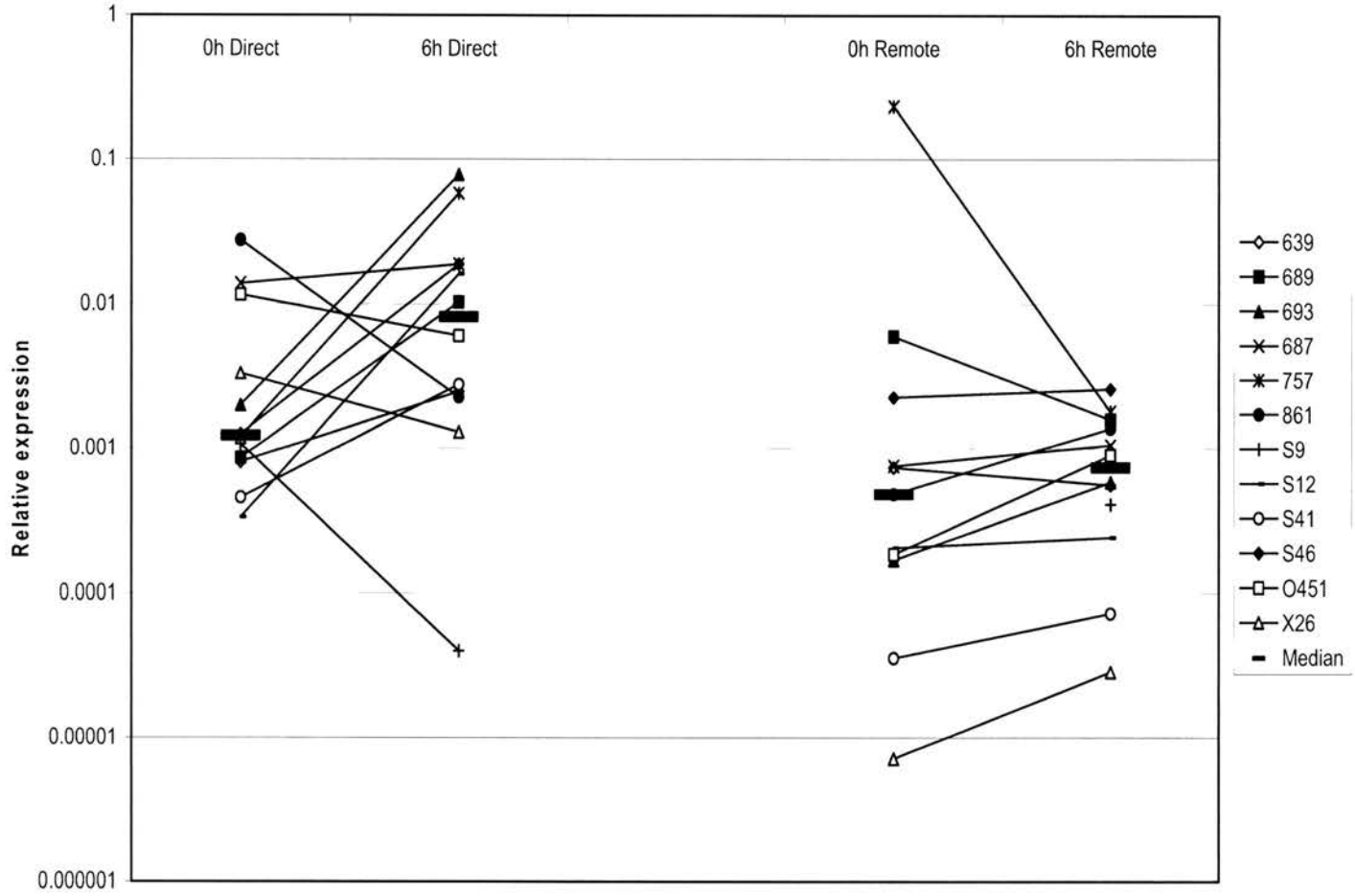


Figure 4.2: Expression level of interleukin (IL) 6 as assessed by real-time RT-PCR analysis of RNA from brushed epithelial cells in challenged (direct) and contra lateral (remote) segments at baseline (0 hours) and post-LPS (6 hours). Expression level relative to ATPase housekeeping gene expression is shown on the y-axis on a logarithmic scale. IL 6 was significantly (p=0.01) increased (4.6 fold mean change)six hours post-LPS challenge in epithelial cells taken from the direct segment. In the epithelial cells taken from the remote segment there was no significant change (p=0.505) in the expression if IL 6.

Tumour necrosis factor α

The relative expression of tumour necrosis factor α mRNA was significantly (p=0.023, n=12) up-regulated six hours post-LPS challenge in epithelial brushings taken from the direct segment (median fold difference relative to baseline: 4.2; range: 0.08-422.4). In epithelial brushings taken from the remote segment there was no significant change (p=0.756, n=11) in the level of TNFα expression. Figure 4.3 shows, for each animal, the change in relative expression of TNFα in epithelial brushings in the directly challenged and remote segments both prior to (0h) and 6 hours after LPS challenge (6h).

Interleukin 8

Interleukin 8 expression was not significantly altered in either the direct (p=1.00, n=12) or remote (p=0.894, n=11) segments six hours after LPS challenge. Figure 4.4 shows, for each animal, the change in relative expression of IL 8 in epithelial brushings in the directly challenged and remote segments both prior to (0h) and 6 hours after LPS challenge (6h).

Interleukin 10

The relative expression of interleukin 10 mRNA in epithelial brushings taken from the direct segment showed no significant change following challenge (p=0.784, n=12). In epithelial brushings taken from the remote segment there was a significant increase (p=0.003, n=10) in the level of IL 10 expression (median fold difference relative to baseline: 10.4; range: 3.3-90.9). Figure 4.5 shows, for each animal, the change in relative expression of IL 10 in epithelial brushings in the directly challenged and remote segments both prior to (0h) and 6 hours after LPS challenge (6h). The overall levels of IL-10 found at 0h in the remote and directly challenged segments show

considerable differences. This overall difference in baseline levels of IL-10 would appear to have no physiological basis, and did not affect the analysis of the cytokine changes in either segment. As the segments chosen were heterogeneous there can be no suggestion of a spatial bias in levels of IL-10 expression at baseline, and there is no correlation with age or sex either, the observation was therefore determined to be artefactual and of no functional or analytical consequence.

The fold changes in relative cytokine expression as a consequence of LPS-instillation are depicted in figure 4.6. Cytokine gene expression at 0h and at 6h was assessed in samples derived from both directly instilled segments (Direct) and contra-lateral segments remote from the site of instillation (Remote).

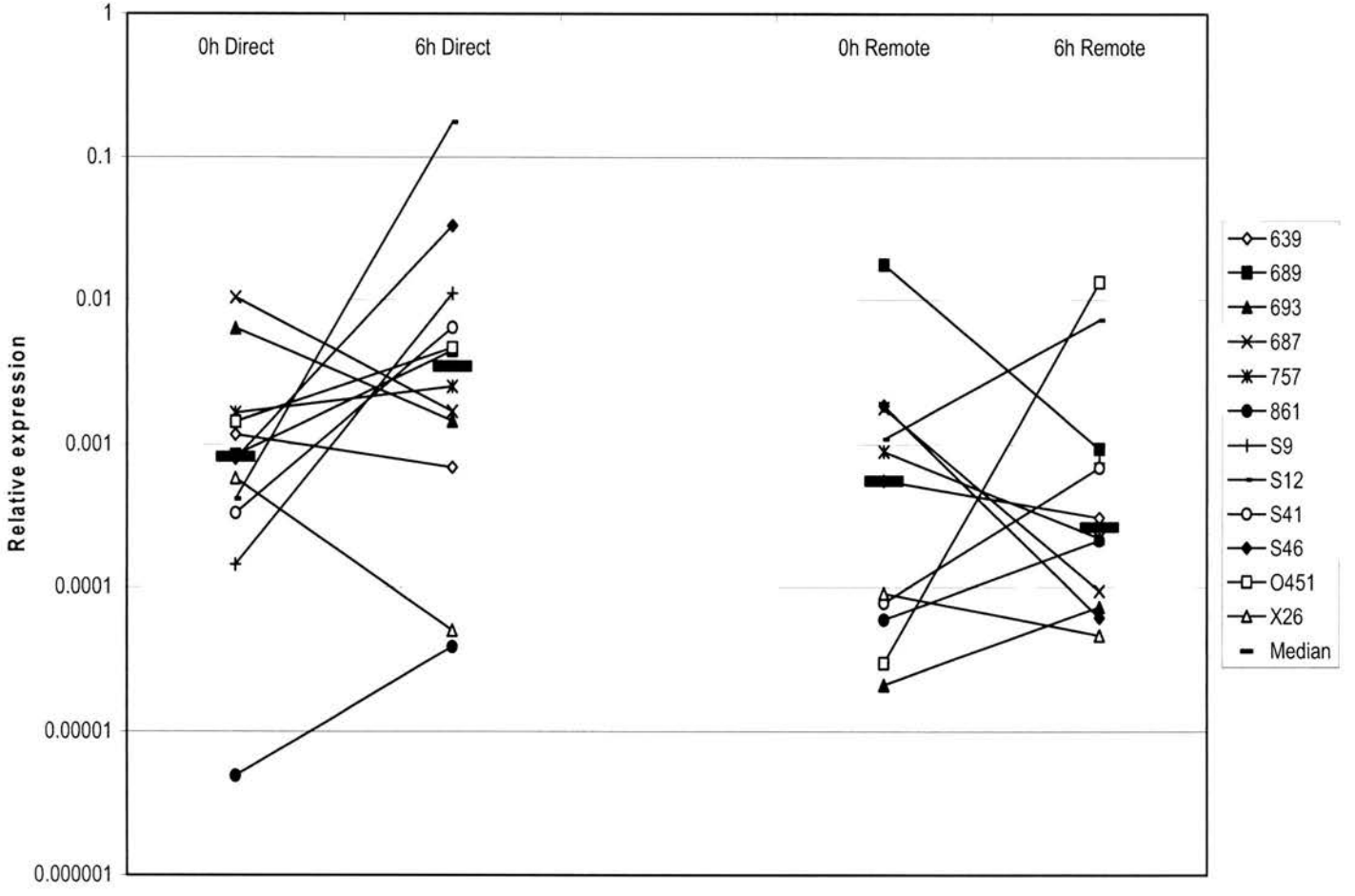


Figure 4.3: Expression levels of tumour necrosis factor α (TNFα) as assessed by real-time RT-PCR analysis of RNA from brushed epithelial cells in challenged (direct) and contra lateral (remote) segments at baseline (0 hours) and post-LPS (6 hours). Expression level relative to ATPase housekeeping gene expression is shown on the y-axis on a logarithmic scale. TNFα was significantly (p=0.023) increased (4.2 fold mean change)six hours post-LPS challenge in epithelial cells taken from the direct segment. In the epithelial cells taken from the remote segment there was no significant change (p=0.756) in the expression if TNFα.

Figure 4: IL8 expression

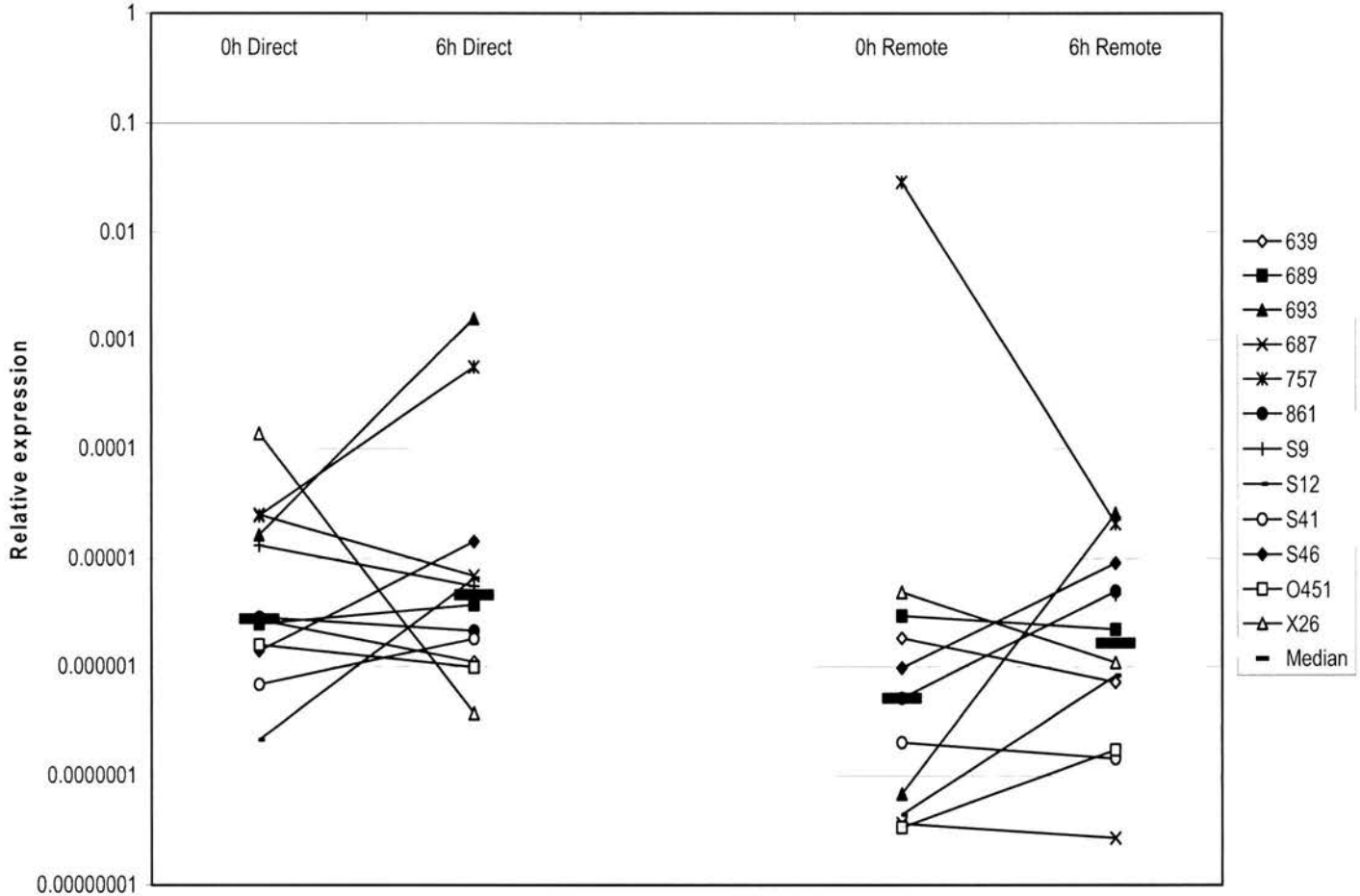


Figure 4.4: Expression level of interleukin (IL) 8 as assessed by real-time RT-PCR analysis of RNA from brushed epithelial cells in challenged (direct) and contra lateral (remote) segments at baseline (0 hours) and post-LPS (6 hours). Expression level relative to ATPase housekeeping gene expression is shown on the y-axis on a logarithmic scale. IL 8 expression was not significantly changed (p=1.0) six hours post-LPS challenge in epithelial cells taken from the direct segment. In the epithelial cells taken from the remote segment there was no significant change (p=0.894) in the expression if IL 8.

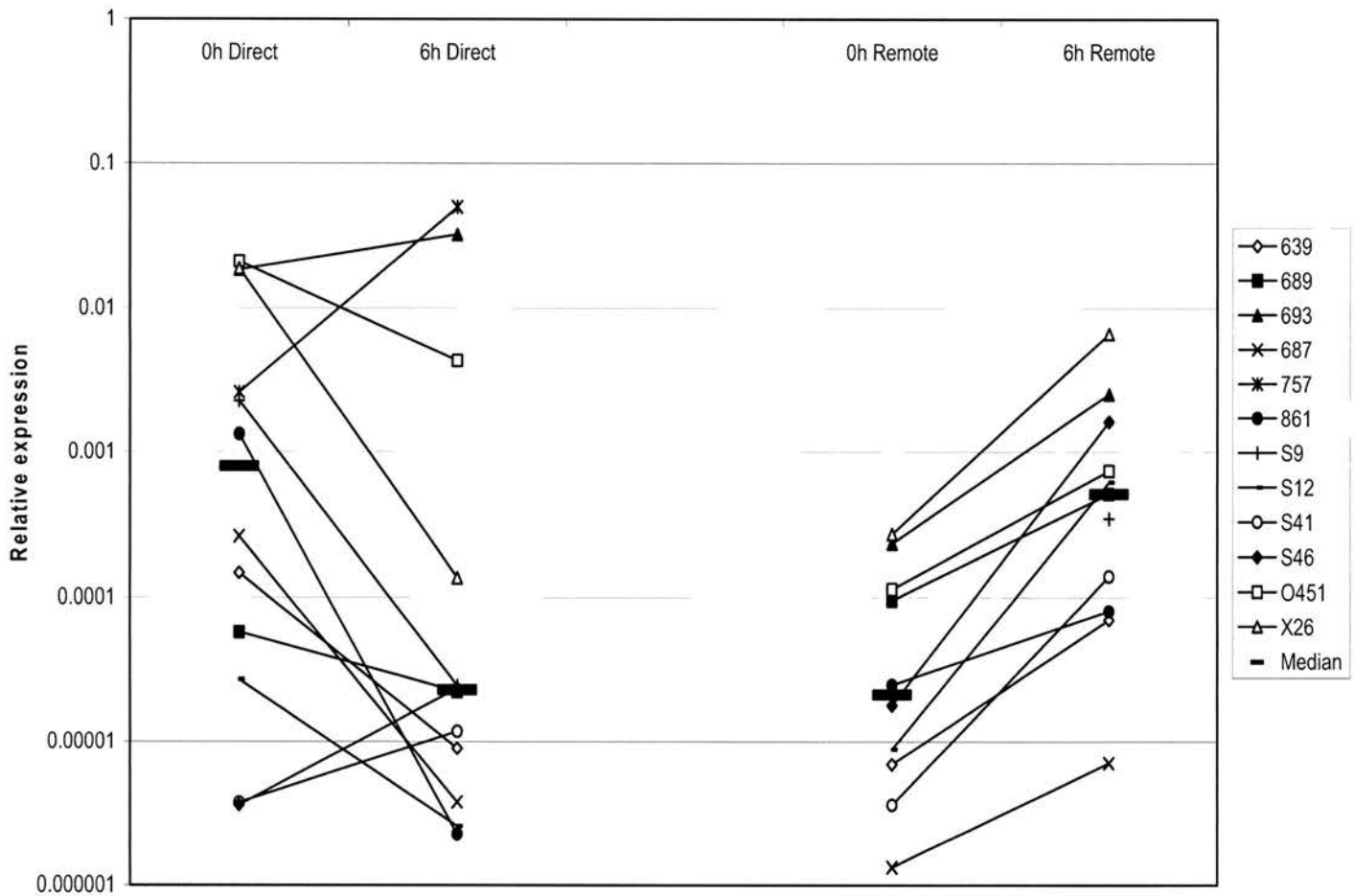


Figure 4.5: Expression levels of interleukin (IL) 10 as assessed by real-time RT-PCR analysis of RNA from brushed epithelial cells in challenged (direct) and contra lateral (remote) segments at baseline (0 hours) and post-LPS (6 hours). Expression level relative to ATPase housekeeping gene expression is shown on the y-axis on a logarithmic scale. IL 10 was significantly (p=0.003) increased (10.4 fold mean change)six hours post-LPS challenge in epithelial cells taken from the remote segment. In the epithelial cells taken from the direct segment there was no significant change (p=0.784) in the expression of IL 10.

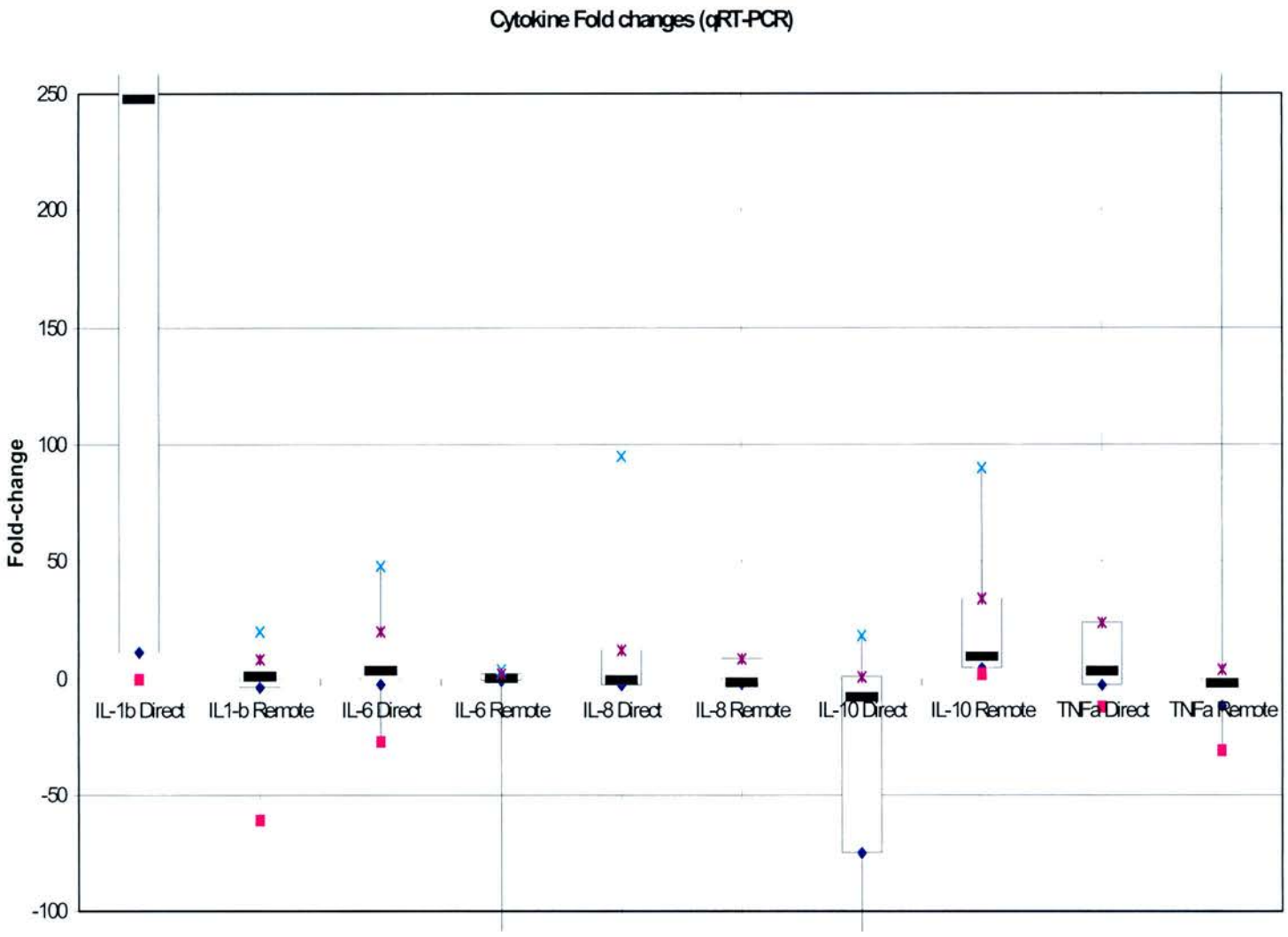


Figure 4.6: Fold changes in expression levels of cytokines in the challenged (direct) and contra lateral (remote) segments post-LPS. Real time RT-PCR determined fold change at 6 hours compared to baseline expression levels is shown on the y-axis. The chart clearly illustrates the changes in expression of IL 1β, IL 6 and TNFα in the direct segment, and IL 10 in the remote segment. The boxes represent the inter-quartile range of expression changes whilst the extended lines represent the overall range. Solid black horizontal bars represent median fold change in expression

Discussion

The described method of brushing the airway wall to obtain epithelial cells suitable for use in molecular assays such as RT-PCR has been reported in humans (Trapnell 1993; Reynolds, Scicchitano, & Holmes 2001; Soria et al. 2002; Muhlebach, Reed, & Noah 2004) but not in the sheep. It is prudent to consider the likely cellular composition of material obtained by this method in sheep. In a study involving human subjects the percentage of ciliated, secretory, and basal-like cells was 24 +/- 2%, 11 +/- 1%, 29 +/- 1%, respectively, while the remaining 36% were difficult to type (Kelsen et al. 1992). This data is consistent with experience in our own laboratory with brushings obtained from sheep (D Collie; personal communication) and is substantiated by a body of literature indicating that the composition of the ovine respiratory epithelium is comparable with that of human subjects (Mariassy & Plopper 1983; Plopper, Mariassy, & Hill 1980).

The quantities of RNA obtained were sufficient to enable samples to be split and half of each used for real-time RT-PCR and the remainder stored for subsequent amplification and use in microarray analyses. Although the limited quantities of sample obtainable by this method precluded standard quality analyses such as the use of denaturing gel electrophoresis the Agilent Bioanalyzer system offers improved resolution of mRNA separation relative to native agarose gels and as such was considered a superior method of analysis of both quality and quantity of RNA samples.

The classic method for determining the quality of RNA is to estimate the ratio of 28S to 18S ribosomal RNA, with a ratio of 2 expected for good quality RNA. Using this criterion the RNA obtained would not have been considered of adequate quality for RT-PCR, however recent publications have made clear that this method does not

correlate in any way with genuine mRNA quality in terms of its suitability for RT-PCR (Miller et al. 2004; Skrypina et al. 2003). That the samples obtained in this study were sufficient in terms of both quality and quantity was evidenced by the consistently early threshold crossing values of the housekeeping genes in the samples used, each of which represented 5% of the total RNA sample collected.

Several reviews indicate that bronchial epithelial cells respond in a predictable fashion to LPS. Following binding of LPS to the phospholipid binding protein, LPS binding protein (LBP), and interaction with soluble or membrane associated LPS pattern-recognition receptors (mannose receptor, the macrophage scavenger receptor (SR-A), collectins, integrins and CD14) a predictable pattern of cellular activation occurs as the innate immune system is triggered (Martin 2000; Toews 2001; Monton & Torres 1998; Takizawa 1998; Levine 1995). Both bronchial epithelial cells and alveolar macrophages contribute to this early innate response through release of pro-inflammatory cytokines, in particular interleukins 1β, 6 and 8 and TNFα(Takizawa 1998). These cytokines activate the resident alveolar macrophages in a positive feedback loop, initiate neutrophil haematopoiesis and release from bone marrow reserves and act, through up regulated expression of adhesion molecules, to localise neutrophils to the challenged area resulting in a rapid and significant localised neutrophilic response (Levine 1995).

At the peak of this T cell independent phase of the innate inflammatory response IL 10, released by epithelial cells, macrophages and other cells, inhibits production of pro-inflammatory cytokines and causes the immune system to switch to the adaptive stage with activation of T-cells and down regulation of the inflammatory response (Toews 2001) This central role for IL 10 in the regulation of the inflammatory

response to LPS and its reported role in the development of ARDS marks it as an important factor to evaluate in terms of any potential remote response.

Whilst the integral role played by IL 12 in the initiation of inflammation is well established in rodent models (Toews 2001) its role in the sheep is poorly characterised. Ovine lung models have demonstrated that the pro-inflammatory cytokines IL 1β, TNFα and IL 6 are consistent markers of gram-negative bacteria-induced inflammation (Murtaugh et al. 1996). Regulation of these cytokines in epithelial cells in primary culture (Crestani et al. 1994) and transformed epithelial cell lines (Koyama et al. 2000) have been characterised in sheep and humans, showing highly analogous regulatory profiles in response to LPS/bacterial challenge whilst epithelial cells from bronchial brushings have been used to quantify cytokine regulation in human subjects following grain dust exposure (Becker et al. 1999).

In the present study real-time RT-PCR (Giulietti et al. 2001) was selected as a quantitative and highly sensitive technique for the measurement of such mRNA transcripts in bronchial epithelial cells. The quantitative measurement of transcript within a cellular sample relies on the quantification of each target relative to an endogenous reference gene that remains unchanged or affected by the experimental challenge, and thus acts as an internal standard (Pfaffl, Horgan, & Dempfle 2002). The method is derived from the original polymerase chain reaction. A standard reverse transcription of RNA to cDNA is followed by an amplification of specific DNA via the use of primer sequences that act to specifically amplify segments of genes or transcripts of interest.

This method is commonly used and accepted as a standard method of transcript detection. The real-time technique adds to this method by measuring the quantity of amplified DNA throughout the reaction. This allows measurement of the level of

DNA to be made specifically at the point of exponential amplification, yielding fully quantifiable information (Schmittgen 2001; Pfaffl, Horgan, & Dempfle 2002).

Previous studies examining the inflammatory responses to bacterial endotoxin and gram-negative bacterial infection in humans (Wesselius et al. 1997), mice (Hirano 1997;Vernooy et al. 2001) and rats (Xing et al. 1994) have shown patterns of cytokine regulation consistent with those seen in the directly challenged segment of the sheep lung in this experiment. The pro-inflammatory effects of interleukin 1β, interleukin 6 and TNFα are recognised (Xing et al. 1999) and accepted as hallmarks of a neutrophil-mediated acute inflammation (Wesselius et al. 1997). In contrast to their well-characterised and familiar roles as macrophage-generated mediators (Mantovani et al. 2001) the roles of these three pro-inflammatory cytokines in the pulmonary epithelium have been relatively poorly documented (Levine 1995).

The pattern of up-regulation of IL 1β, IL 6 and TNFα in ovine lung epithelial cells suggests an important role for epithelial-derived cytokines within the innate immune response to acute bacterial challenge in the lung. Indeed the present findings concur with the wider perception that these cytokines play a central role in mediating inflammatory processes following acute bacterial or endotoxin challenge (Moore & Standiford 1998; Toews 2001). The unknown correlation between mRNA expression and protein production limits any interpretation regarding the physiological effect of the cytokine regulation seen in this investigation, however previous studies in mice (Hirano 1997; Vernooy et al. 2001) and rats (Haddad et al. 2002) have shown strong correlations between cytokine mRNA levels and quantity of protein produced.

Interleukin 1β

Interleukin 1β is one of the two subunits of interleukin 1. IL 1 is a potent pro-inflammatory cytokine that has been implicated in numerous physiological processes

as well as inflammatory diseases (Dinarello 1994). IL 1 is an important mediator of pulmonary inflammation induced by bacteria and bacterial products produced in lungs after administration of LPS, and inhibition of IL 1 activity attenuates lung inflammation caused by LPS (Jordan et al. 2001). Additionally, recombinant IL 1 causes neutrophilic infiltration in the lung comparable to LPS (Ulich et al. 1991). Elevated IL 1 levels have been found in pleural fluids of patients with unilateral community-acquired pneumonia, significantly higher IL 1 concentrations have been measured in bronchoalveolar lavage fluids (BALF) from infected lungs, compared with BALF from uninfected lungs or serum (Crestani et al. 1994).

Clinical investigations into IL 1β expression specifically have indicated significant increases in levels of this cytokine in the tissue compartment and in the BALF of patients with pneumonia compared with control patients (Millo et al. 2004; Wu, Herndon, & Wolf 2003). Moreover, alveolar macrophages recovered from infected lungs spontaneously release more IL 1 into cell culture supernatants than macrophages isolated from the uninfected lung (Dehoux et al. 1994). Evidence from knockout mouse studies and antibody blockade of IL 1 in the mouse have demonstrated that IL 1 is important as a positive mediator of inflammation, however, somewhat in contradiction, its absence results in an increased clearance of bacteria in murine pneumonia models (Rijneveld et al. 2002; Schultz et al. 2002).

Interleukin 6

IL 6 is a pleiotropic cytokine produced locally in the lung tissue and released into the circulation in many situations of immune challenge including endotoxaemia, LPS instillation to the lung, and acute infections (Kishimoto, Akira, & Taga 1992). As well as its role in the immune response to bacterial infections, circulating IL 6, together with pro-inflammatory cytokines TNFα and IL 1, has been shown to be necessary for

the induction of innate immune reactions such as pyrexia, corticorsterone release, and production of acute phase proteins from the liver, including a number of protease inhibitors (Baumann & Gauldie 1994).

This role is usually seen as a homeostatic maintenance mechanism however, it is unclear whether IL 6 is also directly involved in the modulation of inflammation during local or systemic acute inflammatory responses, particularly in relation to modulating cytokine responses and tissue inflammatory infiltration. In a clinical setting serum IL 6 levels appear to correlate with mortality of patients with endotoxic syndrome or trauma and has been considered a marker of severity in bacterial infection (Dehoux et al. 1994; Hack et al. 1989). IL 6 is likely to be produced locally in the lung in response to bacterial products such as LPS, as evidenced by the pattern of localized expression seen in this study which corresponds well with the local regulation observed in unilateral pneumonia (Dehoux et al. 1994).

The difficulty in separating the biologic effect of IL 6 from an ongoing process of tissue injury renders establishing the role of IL 6 as a central factor difficult, and consequently it remains unclear whether it acts as a beneficial or detrimental modulator in an infectious scenario (DiCosmo et al. 1994). Blockade of IL 6 by use of antibodies in LPS models has yielded conflicting information (Barton & Jackson 1993; Heremans et al. 1992; Starnes, Jr. et al. 1990) and although studies in knockout mice have also been contradictory, the balance of evidence appears to suggest a pro-inflammatory role for IL 6 in acute inflammation.

This observation is based on original work by Kopf et al who reported increased susceptibility to infection in IL 6 knockout mice (Kopf et al. 1994). Further investigation by Dalrymple et al (1995) extended this observation by establishing a central role for IL 6 in the rapid initiation of a systemic neutrophilia. Later studies

demonstrated increased susceptibility to *E. coli* infection with concomitant failure to establish blood neutrophilia in mice with otherwise broadly normal expression of other innate immune effectors such as interferon gamma and nitric oxide, and unaffected macrophage and natural killer cell activation (Dalrymple et al. 1995). Establishing the functional role of IL 6 in bacterial inflammation remains a key target in inflammatory studies, however it is known to exert both pro-inflammatory effect in acute inflammation, and anti-inflammatory effects in chronic models (Xing et al. 1998) and to be an important balancing cytokine in inflammatory disease models (Aderka, Le, & Vilcek 1989; Appelberg 1994; Chen et al. 1993; Schindler et al. 1990).

Tumour necrosis factor α

Tumour necrosis factor α is mostly produced by macrophages and monocytes though other cell types in the airway have the ability to generate TNFα including eosinophils (Costa et al. 1993; Finotto et al. 1994) epithelial cells (Khair et al. 1994) and mast cells (Thomas 2001). TNFα activates neutrophils and macrophages leading to production of cytokines and immune mediators, as well as directly enhancing bactericidal activity and phagocytosis (Strieter, Kunkel, & Bone 1993; Le & Vilcek 1987). TNFα is also a chemotactic cytokine for granulocytes including eosinophils and neutrophils (Ming, Bersani, & Mantovani 1987) this is achieved through up-regulation of cellular adhesion molecules such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1or CD54) (Gamble et al. 1985; Pober et al. 1986).

Tumour necrosis factor α induction of adhesion molecules on pulmonary endothelium has been demonstrated as an important factor in the recruitment of granulocytes

(Yamamoto, Sedgwick, & Busse 1998). TNFα has been found to be expressed in lungs of clinical patients with bacterial pneumonia (Nohynek et al. 1991) as well as having been reported to play a critical role in clearance of streptococcal pneumonia in a mouse model (van der et al. 1997). Other murine models of gram negative pneumonia have also demonstrated the central role of TNFα in bacterial clearance from the lungs, and blockade of TNFα in these models has resulted in increased mortality and impairment of bacterial clearance (van der et al. 1997; Takashima et al. 1997; Laichalk et al. 1996; Gosselin et al. 1995).

Interleukin 8

In contrast to the significant up regulation of IL 1β, IL 6 and TNFα in direct segments, no significant change in the expression of IL 8 occurred following challenge with LPS. . Several studies have examined the cytokine response of the A549 transformed human type II-like bronchial epithelial cell line to challenge with pure LPS (Ahmad et al. 2003; Palmberg et al. 1998), grain dust (Palmberg et al. 1998; Jagielo et al. 1996), swine dust (Palmberg et al. 1998) or immune modulators or cytokines (Ahmad et al. 2003; Standiford et al. 1996). The role of IL 8 in such systems is not clearly defined in that Standiford (Standiford et al. 1990) demonstrated that the A549 cell line does not produce IL 8 directly as a response to LPS challenge whereas Ahmad (Ahmad et al. 2003) concluded that, in the presence of immune serum, IL 8 is indeed produced.

Primary cell culture experiments using LPS challenge are similarly contradictory with one study showing no change in IL 8 mRNA levels (Pechkovsky et al. 2000) and another indicating significant up-regulation (Palmberg et al. 1998) following challenge with both LPS and swine dust respectively. Nevertheless the literature indicates that levels of IL 8 protein in BALF (Jagielo et al. 1996) and IL 8 mRNA

levels in epithelial cells (Jagielo et al. 1996) are both raised following instillation of LPS to the lungs of rodents.

The time-course of this response is unclear, and no assessment of IL 8 mRNA in ovine bronchial epithelial cells has been previously reported *in vivo*. The stimulatory effect of LPS on alveolar macrophages to produce IL 8 along with other pro-inflammatory cytokines is clear from published studies (Standiford et al. 1990; Strieter et al. 1990), and authors have proposed that the production of IL 8 from epithelial cells may be as a result of this production, a hypothesis supported by the lack of appropriate receptors for LPS-stimulated IL 8 production on epithelial cell surfaces in comparative species (Wright et al. 1991; Wright et al. 1990b; Wright et al. 1990a).

Interleukin 10

Interleukin 10 is released from alveolar macrophages and epithelial cells as part of the late phase acute inflammatory response (Standiford et al. 1995). Its role relates to the down-regulation of inflammation by inhibition of pro-inflammatory cytokine release (Thomassen, Divis, & Fisher 1996) through blockade of the phospholipase C signal pathways (Lo, Fu, & Cryer 1998) and NF-kB transcriptional pathway (Raychaudhuri et al. 2000). IL 10 has been shown to inhibit endotoxin-mediated TNFα, IL 1β, IL 6 and IL 8 release from alveolar macrophages (Thomassen, Divis, & Fisher 1996) as well as neutrophil chemotaxis (Dokka et al. 2000) and mortality due to LPS sepsis (Inoue 2000).

Abrogation of the IL 10 response has been implicated in the development of acute respiratory distress syndrome (Standiford et al. 1995) and has been shown to cause systemic LPS induced shock (Ishida et al. 1994), enterocolitis (Kuhn et al. 1993) and increased lethality in endotoxaemia (Standiford et al. 1995). A significant ten-fold

increase in IL 10 gene expression occurred in the remote segment six hours after LPS challenge. This anti-inflammatory response remote from local endotoxin challenge has not been reported in any tissue or in any species. Indeed remote responses of any character have yet to be reported in lung tissue at the molecular level.

It could be speculated that the role of remotely produced IL 10 may be a protective response against systemic, lung-wide inflammation. Results from previous studies (Raychaudhuri et al. 2000) would suggest that the increased expression of IL 10 would serve to down-regulate any inflammatory mediators and inactivate any immune cells that were to infiltrate the pulmonary space.

The systemic neutrophilic leukocytosis that occurs as a consequence of local lung instillation of LPS could conceivably lead to an increase in the number of primed and/or activated granulocytes trafficking through lung tissues remote from the site of inflammation. Remote up-regulation of IL 10 could potentially serve as a mechanism for limiting damage to the organ as a whole.

Overview

Overall this study confirmed the expression profile of the 'classical' inflammatory cytokines IL 1β, IL 6 and TNFα in unilateral LPS instillation after 6 hours post-instillation. There are no previously published examples of this model of inflammatory lung disease, and the profile of cytokine expression in the affected segment is closely analogous to that seen in unilateral pneumonia both clinically and in other animal (particularly rodent) models of acute lung injury, pulmonary endotoxaemia and pneumonia. The remote expression of IL 10 in the lung is a novel observation. Previous studies including investigation of the contra lateral lung have been limited to phenotypic and cellular changes, with some including measurements of the protein levels of classical pro-inflammatory markers in bronchoalveolar lavage

fluid. The observed increase in IL 10 also provides a mechanism for the observed hyporeactivity of alveolar macrophages isolated from bronchoalveolar lavages derived from patients with unilateral pneumonia as this cytokine has been demonstrated to be a potent suppressor of macrophage function *in vitro* (Lo, Fu, & Cryer 1998; Raychaudhuri et al. 2000).

The consequences of the observed anti-inflammatory gene expression in remotely situated lung tissue following unilateral localised LPS inflammation may relate to the balance of inflammatory regulators present in the systemic circulation and tissue compartments, and this balance may play an important role in subsequent infection. This is of particular note with regard to potential impact on the development of diffuse lung injury, further bacterial colonisation of the lung and the progression to ARDS. IL 10 has been shown to impair PMN function both *in vivo* and *in vitro*, as well as playing a role in the blockade of PMN infiltration and sequestration in the tissue compartment (Dokka et al. 2000; Inoue 2000; Quinn et al. 2000).

IL 10 expression in tissue has been implicated in increasing the vulnerability of the lung to bacterial colonisation and bacterial outgrowth in pulmonary infection (Greenberger et al. 1995; Reddy et al. 2001; Steinhauser et al. 1999). The effects of IL 10 in the tissue compartment have been detrimental in terms of clearance of infectious organisms and in terms of survival following infection (Sewnath et al. 2001; van der et al. 1996). These effects in tissue are in contrast to the documented effects of IL 10 in the systemic circulation where its impact is largely protective (van der et al. 1995). Studies in IL 10 knockout mice showed improved clearance of bacteria from the lung – presumably due to improved inflammatory ability within the pulmonary space – but reduced survival indicating a central protective role in the resolution of infection for systemic IL 10.

During primary pneumonia with no subsequent or prior inflammatory stimulus the balance between pro- and anti-inflammatory responses in the lung functions as a highly effective mechanism to clear bacteria whilst protecting the lung from subsequent tissue destruction by over-production and tissue sequestration of PMNs. However in the unusual situation where this primary inflammatory stimulus is followed by a secondary infection the anti-inflammatory response may become a liability and a potential window of innate immuno suppression may then allow colonisation and outgrowth of bacterial pathogens and consequent morbidity and mortality. This window of vulnerability would be particularly important if the levels of remote IL 10 in tissue stayed high whilst the systemic IL 10 levels dropped back to baseline levels, and it is conceivable that this set of circumstances predisposes for ARDS in the clinical setting. Indeed such a notion correlates well with observations in pulmonary ARDS patients linking secondary infection with development of the syndrome.

As there has been no study revealing this remote anti-inflammatory response previously, this hypothesis has yet to be challenged, however it concurs with a study involving trauma patients in which systemic and alveolar IL 10 levels were investigated (Muehlstedt, Lyte, & Rodriguez 2002). In this study patients admitted with traumatic injury were assessed for susceptibility to nosocomial pneumonia. Levels of IL 10 were measured systemically and in alveolar spaces both at admission (pre-infection) and at later time-points. Systemic IL 10 levels in patients who acquired pneumonia and those who remained free from pneumonia followed similar temporal profiles, whilst those that developed pneumonia showed significantly higher alveolar levels of IL 10 than those that did not.

The implication of this study closely mirrors the assertion above that the systemic: tissue balance in anti-inflammatory (IL 10) expression is a critical factor in the susceptibility of the lung to bacterial invasion. In addition to the potential for identification of potential avenues of intervention in the development of ALI/ARDS, it is also conceivable that this window of opportunity could be manipulated in unrelated pulmonary applications such as gene therapy as a means of circumventing the negative effects of immune reactions to applied treatments.

This investigation establishes for the first time the validity of a real-time PCR approach to investigating cytokine responses to segmental LPS challenge in the ovine lung. A number of variants of the real-time technique exist, with probe-based and intercalating dye-based methods predominant. Probe based techniques use a labelled probe specific to a portion of the amplified section of DNA. When bound as double stranded DNA the probe fluoresces. This method is highly specific due to the combined specificity of the primer pair and the labelled probe however it suffers from disadvantages, particularly related to high cost, time delays and poor sensitivity (Bustin 2002).

The intercalating dye method uses a usually inert dye (e.g. SYBR green) that, when bound to double stranded DNA, fluoresces at a known wavelength (Lekanne Deprez et al. 2002). An important advantage of this more sensitive technique is that the dye can be used for detection of any amplified transcript with no need for probe design or purchase. However, a major disadvantage of the technique is the loss of sensitivity as the dye can bind to any non-specifically amplified DNA as well as any primer-dimers that form through normal molecular interactions (Vandesompele, De Paepe, & Speleman 2002). As a result this latter method requires considerable optimisation compared with probe-based techniques.

The characterisation of this remote response due to LPS inflammation confirms the hypothesis that the ovine lung will respond on a whole-lung basis to local inflammation. Further investigation to fully characterise the nature of this remote response may potentially lead to novel avenues of investigation and even prophylaxis in terms of the manipulation of whole organ responses to acute onset localised gram-negative sepsis in the lung.

The immediate investigation into the remote response will concentrate on the expression profiles of a large cohort of sequences in the lung in order to assess the extent of the anti-inflammatory reaction in this segment as well as to fully characterise the networks of genetic changes that underlie this event. This characterisation may provide clues as to a potential mechanism that drive the remote changes in response to LPS inflammation and thus expose avenues of intervention and further investigation. At this preliminary stage it is unlikely that large-scale genomic analysis will provide complete answers to the questions concerning the fundamental nature of the remote response to local inflammatory stimulus, but is instead a springboard toward further investigative directions in that regard.

Additional investigation will seek to establish the effect of the identified response at 6 hours through a secondary challenge in the contra-lateral lung. This investigation may give an indication of any overt alteration in the ability of the lung to initiate inflammatory responses at the phenotypic level following pre-conditioning of a contra-lateral segment. A logical working hypothesis would be that this pre-conditioning would result in attenuation of the response to LPS, and may induce a form of endotoxin tolerance similar to that seen *in vitro* following both pro- and anti-inflammatory stimulus.

In conclusion, the assessment of cytokine gene expression profiles described has shown, for the first time that the lung responds in a whole organ manner to localised LPS-induced inflammation. Further it has shown that the lung promotes an anti-inflammatory reaction remote from the focus of inflammation, which significantly expands upon the available body of knowledge as concerns the inflammatory response to bacterial infection in the lung.

Chapter 5 Gene discovery and microarray production

Aims and Objectives

- To assemble an array of cDNA elements comprising those ovine genes that are preferentially expressed in the lungs and a selection of more widely expressed highly homologous bovine genes.
- To fabricate a microarray of these elements capable of profiling the expression of gene transcripts expressed in bronchial epithelial cells in response to LPS instillation in the ovine lung.

Introduction

With the objective of characterising the molecular response of the ovine lung to an inflammatory stimulus and no *a priori* assumption as to the characteristics of that response it was assumed that the novel cDNA microarray should ideally be capable of analysing gene expression from as wide a pool of genes as possible. Indeed the elements on the array should represent commonly expressed and highly abundant gene products as well as lung specific, differentially expressed and rare transcripts.

In order to generate a set of elements representing the high abundance and more commonly found gene products, an approach involving the use of bovine clones was devised. Known and characterised ovine sequences from the NCBI database were used to query the collected sequences from an available bovine genomic library, with clones showing suitably high homology with ovine sequences then being used to represent those ovine products on a microarray.

In order to discover elements representing lung specific transcripts, including those expressed at low levels, a subtractive suppressive hybridisation (SSH) library

technique was employed. The SSH technique involved subtracting pooled mRNA from heart, brain, liver, kidney and skeletal muscle tissues from lung-derived mRNA and then normalising the abundance of the sequences in the subtracted population to enrich for rare transcripts. The two populations of clones (SSH and bovine) were then printed onto prepared glass slides and these microarrays were used to probe the differential expression pattern between bronchial brushing RNA samples in the directly challenged and contra lateral lung segments. These hybridisations were also analysed to assess both the functionality of the array and the success of the SSH experiment.

Results

Identification of ovine homologs to bovine clones.

The BLAST searches of ovine genes against the MARC bovine library clone set yielded a total of 4,952 clones with a greater than 70% homology with their respective ovine sequences. There was considerable redundancy within these clones, with a total of 524 unique ovine sequences represented (mean 9.5 times redundancy). Clones showing significant overlap within each sequence were assessed and those showing greater overall homology retained for inclusion. The final list of clones selected for inclusion comprised 1,694 clones (mean 3.2 fold redundancy). The mean homology in this set was 90.75% (S.D. 7%). The lists of all homologs identified and the final clones used in the array are detailed in appendix III.

Suppressive subtractive hybridisation library

Subtraction

2 µg mRNA was purified from pooled tissue samples of lung, heart, brain, liver, kidney and skeletal muscle from six adult sheep, this quantity was derived from large

samples of total RNA and diluted to generate a representative mRNA sample conforming to the quantity required by the SSH experimental process. The RNA was reverse transcribed to cDNA and digested with Rsa I resulting in smaller fragments as shown in Figure 5.1a. Following Rsa I digestion the cDNA was ligated to adaptors 1 and 2R the results of which are shown in a gel image in Figure 5.1b.

The adaptor-ligated sequences were hybridised twice and then underwent two rounds of nested PCR. After each round of PCR the population of cDNA was size selected for sequences greater than 200bp length. Figure 5.1c-d shows the results of these PCR rounds. It can be seen that the subtracted lanes contain lower molecular weight bands than in the unsubtracted lanes. The second round of PCR also accentuated the bands standing out from the general smear. The control lanes clearly show that human skeletal muscle control samples have also been subtracted as intended indicating the success of the process.

Cloning

The pool of subtracted cDNA was cloned into pCRII vector using the TOPO TA cloning kit, and then transformed into TOP10F *E. coli* bacteria. These were grown overnight on Kanamycin LB agar plates and in LB broth with kanamycin. Table 5.1 lists the numbers of clones counted at the various dilutions of sample. The cloning appeared to be initially successful and the confluent liquid cultures of bacteria were then serially diluted and grown on LB Kan^+ plates, the results of this are shown in table 5.2. From these counts a titre of 1.66×10^6 c.f.u. / µl was obtained. An aliquot of this library was sent to ARK genomics for colony picking and sequencing.

In addition to the aliquot sent for sequencing, an initial screen was performed on 40 of the clones obtained from the initial growth of the library. This screen involved picking

*Table 5.1: Details of the numbers of white (positive) and blue (negative) clones appearing on the agar plates after the cloned SSH library was spread at various concentrations. Positive controls were a previously assessed plasmid transformed into the same bacteria and using the same method as for the experimental SSH clones. Negative controls were reactions using empty plasmid. The plates used contained IPTG allowing blue / white screening to differentiate between clones carrying insert-bearing plasmids (white) and those not (blue). Control reactions resulted in the expected percentages of positive clones. The SSH reaction had a higher proportion of negative (blue) clones than had been expected according to the manufacturer's claims, and this was initially considered to be due to a lower efficiency caused by the use of a mixed DNA population in the cloning reaction. Subsequent screening indicated that a number of blue clones in fact contained inserts and this therefore implied failure of the blue / white screen in this experiment. Samples labelled * were extrapolated from counts of quarter sections of the plate.*

Sample	White colonies	Blue colonies	Positive %
Control - (100µl)	5	60	8
Control - (50µl)	0	54	-
Control + (100µl)	500*	50	91
Control + (50µl)	300*	24	93
SSH (100µl)	280	140	67
SSH (50µl)	73	61	55
SSH (25µl)	40	26	61

*Table 5.2: Details of white (positive) and blue (negative) clones appearing on the agar plates after 100 µl of each serially diluted concentration of cells from the cloned SSH library was spread. Negative controls were reactions using untransformed bacteria. The plates used contained IPTG allowing blue / white screening to differentiate between clones carrying insert-bearing plasmids (white due to disruption of X-gal gene) and those not (blue due to hydrolysis of IPTG to blue dye). Control reactions resulted in a higher than expected percentage of positive clones. The SSH reaction had a slightly higher proportion of negative (blue) clones than had been expected from the manufacturer's claims, and this was initially considered to be due to a lower efficiency caused by the use of a mixed DNA population in the cloning reaction. Subsequent screening indicated that a number of blue clones in fact contained inserts and this implied failure of the blue / white screen in this experiment. Sampled labelled * were extrapolated from counts of quarter sections of the plate. The sample labelled ** was uncountable as the clones present were so numerous as to form a continuous lawn.*

Dilution	White colonies	Blue colonies	Positive %
$1 / 1 \times 10^{10}$	0	2	0
$1 / 1 \times 10^{8}$	4	6	40
$1 / 1 \times 10^{6}$	53	30	64
$1 / 1 \times 10^{4}$	300*	200*	60
$1 / 1 \times 10^{2}$	**	**	**
Control (0 cells)	22	131	14

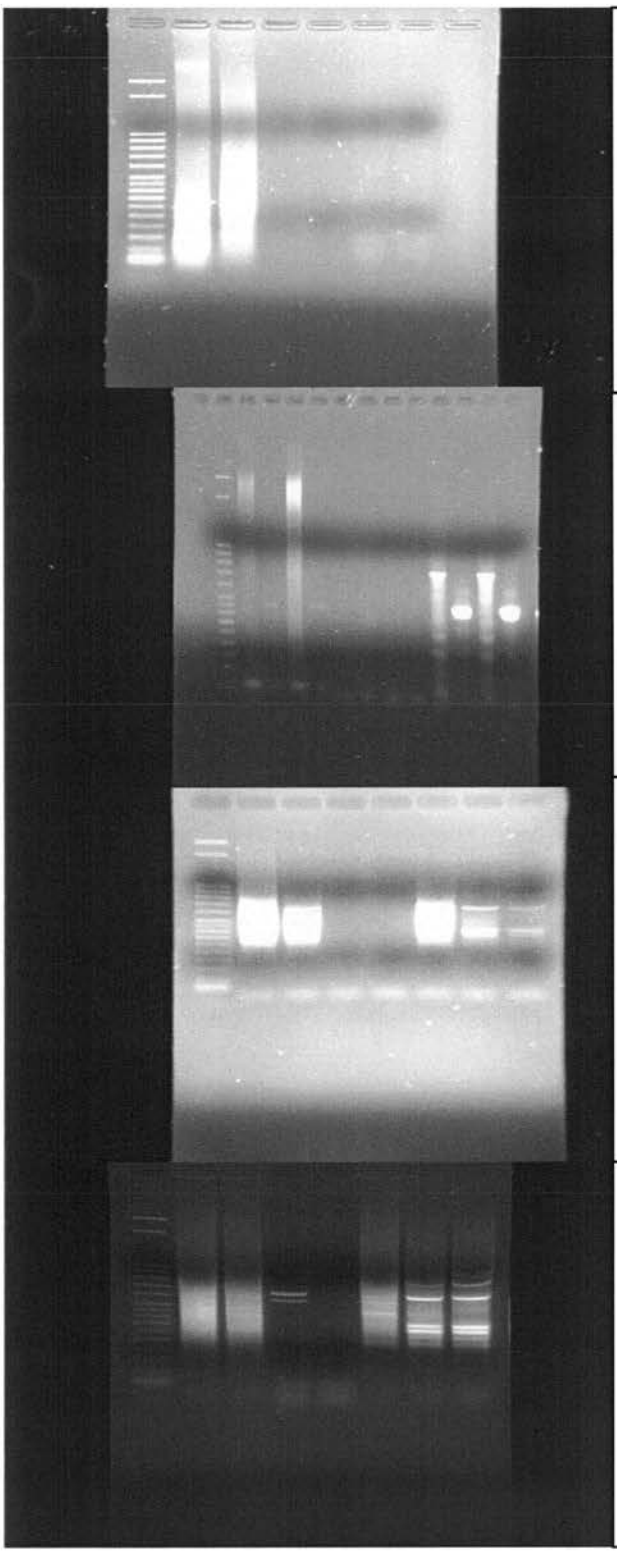


Figure 5.1.A.: Gel electrophoresis analysis of the Rsa1 digestion stage of the SSH procedure. From left to right - Lanes:
1: Molecular weight marker(MWM).
2: Undigested driver (pooled organs).
3: Digested driver (pooled organs).
4: Undigested tester (lung).
5: Undigested tester (lung).
6: Digested control (human muscle).
7: Undigested control (human muscle).

Figure 5.1.B.: Gel analysis of the adaptor ligation step of the SSH procedure. From left to right - Lanes: 2: MWM.
3-6: GAPDH driver assessment. (human GAPDH primers, pooled sheep organs)
7-10: GAPDH tester assessment (human GAPDH primers, sheep lung).
11-14: GAPDH control assessment (human GAPDH primershuman muscle).

Figure 5.1.C.: Gel analysis of the first PCR subtraction stage of the SSH procedure. From left to right - Lane 1: MWM.
2: Unsubtracted forward (total sheep lung)
3: Subtracted forward (pooled from lung)
4: Unsubtracted reverse (total pooled)
5: Subtracted tester (lung from pooled)
6: Unsubtracted control (human muscle)
7: Subtracted control (subtracted muscle)
8: Positive control (subtracted muscle)

Figure 5.1.D.: Gel analysis of the second PCR subtraction stage of the SSH procedure. From left to right - Lane 1: MWM.
2: Unsubtracted forward (total sheep lung)
3: Subtracted forward (pooled from lung)
4: Unsubtracted reverse (total pooled)
5: Subtracted tester (lung from pooled)
6: Unsubtracted control (human muscle)
7: Subtracted control (subtracted muscle)
8: Positive control (subtracted muscle)

Figure 5.1: Fig A shows the change in size between the digested and undigested samples, demonstrating successful digestion. This was true across the samples, though the concentration difference between samples rendered visualisation of all samples simultaneously impossible. Fig B shows the amplification of a housekeeping gene in each sample following adaptor ligation. The primers used were human, resulting in poorer amplification from the ovine samples. Figs C and D are the analyses of the subtraction hybridisations. The clearer bands in the subtracted samples indicate the successful elimination of common fragments.

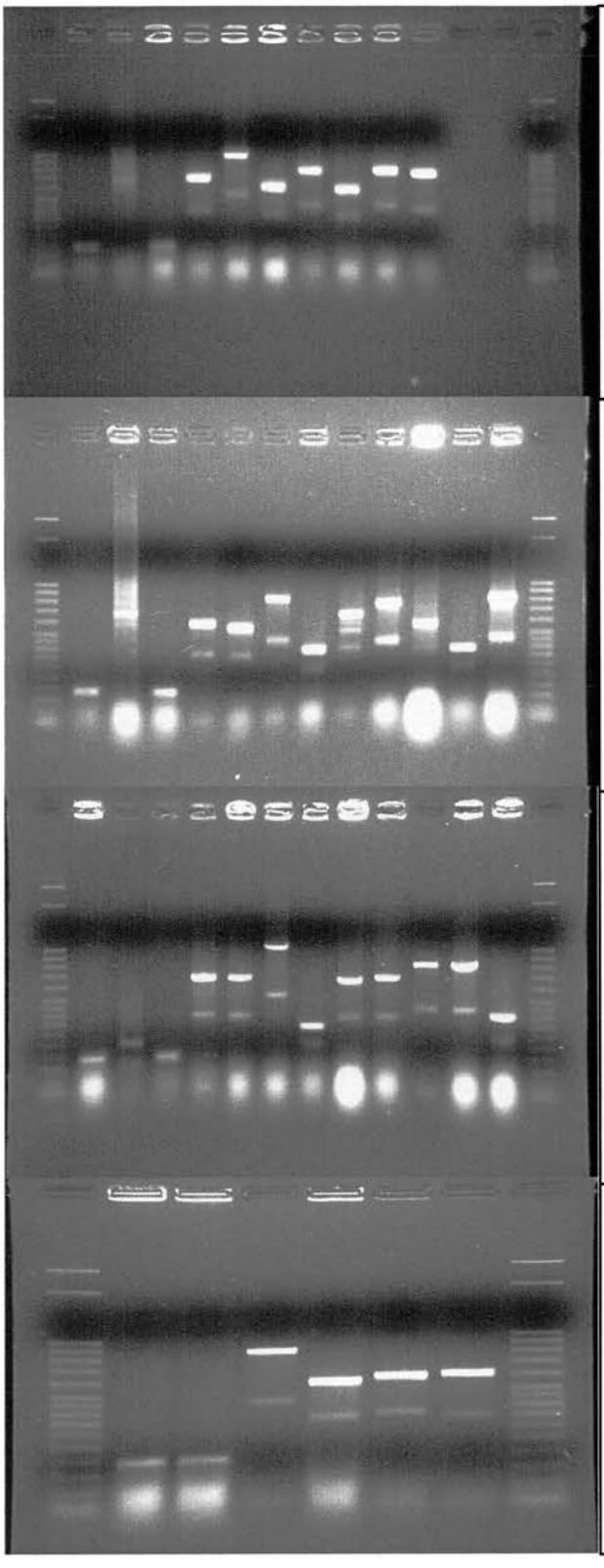


Figure 5.2i: Gel electrophoresis analysis of the PCR products derived from the screening of SSH clones. Lanes from left to right. 1+14: molecular weight markers; 2: plasmid only negative control; 3: previously screened positive control colony; 4: colony from negative control plate; 5-11: white SSH colonies; 12-13: empty

Figure 5.2ii: This is an image of gel electrophoresis analysis of the PCR products derived from the screening of SSH clones. Lanes from left to right. 1+14: molecular weight markers; 2: plasmid only negative control; 3: previously screened positive control colony; 4: colony from negative control plate; 5-13: white SSH colonies.

Figure 5.2iii: This is an image of gel electrophoresis analysis of the PCR products derived from the screening of SSH clones. Lanes from left to right. 1+14: molecular weight markers; 2: plasmid only negative control; 3: previously screened positive control colony; 4: colony from negative control plate; 5-13: white SSH colonies.

Figure 5.2iv: This is an image of gel electrophoresis analysis of the PCR products derived from the screening of SSH clones. Lanes from left to right. 1+8: molecular weight markers; 2: plasmid only negative control; 3: colony from negative control plate; 4-7: blue SSH colonies.

Figure 5.2: The PCR screening of the colonies grown from the SSH library cloning procedure established that the overall SSH process was a success. The screened white colonies displayed a variety of product sizes demonstrating insertion of multiple fragments of different sizes. This resulted in a cloned library representing many varied sequences in the plasmids. Control samples amplified successfully, though more weakly than experimental ones. Blue colonies were also shown to contain inserts (Figure 5.2.iv), implying underestimation of the positive clone numbers.

clones directly into a PCR mix using the sequencing primers corresponding to the appropriate part of the cloning vector. The results were run on agarose gels, which are shown in Figure 5.2. This initial screen indicated that approximately 80% of the white colonies obtained were positive for inserts with approximately 33% variation in size between the positive clones. It also indicated that around 20% of the blue colonies were false negatives.

Sequencing

The library sent to ARK was plated onto multi-well bio-plates and the clones that were grown were picked to select white colonies. These were then subjected to a preliminary screen in order to select clones with a genuine insert. 238 of the positive clones from the initial screen were sequenced using an ABI robotic scanner and the results returned for analysis. The sequences were trimmed to remove vector sequences flanking the insert, and the trimmed sequences assembled into the contig groups shown in Table 5.3.

These groups were clusters of similar sequences, with an 80% minimum homology threshold for inclusion, from which a consensus sequence was derived for each group. As the majority of the contig groups contained only a single sequence or pair of similar sequences it is likely that the number of sequences obtained has not identified each of the individual sequences present within the population and that there are therefore likely to be a number of additional sequences within the library yet to be discovered. The 100 consensus sequences were assessed for any remaining vector contamination and then run through an initial BLAST search against the NCBI EST database.

Table 5.3: Representative SSH sequences aligned into contig groups along with the length of the consensus sequence derived from each contig alignment. 234 sequences were aligned in total in two batches. The first batch (table 5.3.A) yielded 25 contig groups from 96 original sequences, representing approximately 3.8-fold redundancy. The second batch (table 5.3.B), selected from significantly regulated sequences identified from microarray analysis, yielded 75 contig alignments from 138 original sequences, representing approximately 1.8-fold redundancy. The different amounts of overlap in the two batches was probably due to the selection of variably expressed sequences from the array data. Overall the redundancy within the evaluate SSH library sequences was 2.34-fold. The lengths of consensus sequences are consistent with those seen in publicly available EST libraries produced by various methods.

Table 5.3.A: First batch of SSH contig alignment groups.

Contig Number	Length (consensus)	Number of Sequences
1	758	19
2	410	15
3	704	2
4	514	8
5	580	5
6	679	5
7	689	4
8	406	2
9	418	1
10	423	1
11	420	1
12	425	1
13	641	1
14	500	1
15	471	1
16	399	1
17	671	1
18	638	1
19	576	12
20	504	1

21	871	2
22	327	2
23	709	1
24	410	1
25	510	1

Table 5.3.B: Second batch of SSH contig alignment groups.

Contig Number	Length (consensus)	Number of Sequences
1	1027	13
2	911	3
3	840	2
4	513	17
5	933	1
6	944	2
7	475	1
8	862	1
9	479	3
10	676	1
11	912	10
12	922	1
13	926	1
14	886	2
15	894	1
16	877	1
17	463	1
18	327	2
19	218	1

20	384	1
21	824	1
22	246	1
23	945	2
24	856	1
25	242	1
26	837	1
27	531	2
28	351	1
29	629	1
30	385	1
31	896	1
32	382	1
33	759	1
34	541	1
35	657	1
36	895	1
37	998	5
38	910	1
39	273	1
40	819	1
41	416	2
42	335	1
43	361	1
44	448	1
45	920	5
46	941	1

47	980	1
48	912	2
49	421	3
50	977	1
51	955	1
52	1012	1
53	982	1
54	1005	1
55	931	1
56	1066	1
57	1017	1
58	971	1
59	1016	1
60	285	1
61	581	1
62	275	1
63	942	1
64	403	1
65	533	1
66	481	1
67	991	1
68	485	1
69	433	1
70	1048	1
71	938	1
72	432	1
73	401	1

74	496	2
75	488	1

Table 5.4: Homologs found corresponding to each consensus sequence from batch 1 (table 5.4.A) and batch 2 (table 5.4.B) representing the contig group listed. For each sequence a general database search both against the NCBI database and the TIGR cluster database was conducted in order to attempt a tentative identification of the gene from which the sequence originally came. Clones which did not show homology after this were translated to protein sequences and an identification attempted on that basis. Those that could not be identified were labelled as unknown.

Table 5.4.A: First batch of SSH contig alignment groups.

Contig	EST hit	Description	Tentative ID
1	AW427705	64054 MARC 3BOV Bos taurus cDNA 5'	Unknown
2	CB421002	593997 MARC 6BOV Bos taurus cDNA 5'	Unknown
3	AW427705	64054 MARC 3BOV Bos taurus cDNA 5'	Unknown
4	BI775330	467760 MARC 2BOV Bos taurus cDNA 5'	Sorbin
5	CB419863	592757 MARC 6BOV Bos taurus cDNA 3'	Gonadal PISRT1
6	CF117165	Fp290.z2 fp 73-75d foetal sheep skin library Ovis aries cDNA	Ribosomal protein L23a
7	AW659822	97634 MARC 1BOV Bos taurus cDNA 5'	Ribosomal protein
8	CB450615	705290 MARC 6BOV Bos taurus cDNA 3'	Thymosin beta 4
9	CD285973	10_A14.abd POE14_(Day_14_pregnant_ovine _endometrium) Ovis aries	Ribosomal protein S16
10	CD286750	13_F8.abd POE14_(Day_14_pregnant_ovine _endometrium) Ovis aries	Ubiquitin-like/S30 ribosomal fusion protein
11	BM446397	1IL7C3.ab1 Bos taurus Ileum #1	Ubiquinol-cytochrome c

		library Bos taurus cDNA	reductase binding protein
12	BI681605	461037 MARC 1BOV Bos taurus cDNA 5'	Carbonic anhydrase III(Carbonate dehydratase III) (CA- III).
13	CB168659	VQT602700466.R1 CSEQFXL06 stomach-reticulum Bos taurus cDNA	NADH-ubiquinone oxidoreductase chain 2.
14	CB465370	726643 MARC 6BOV Bos taurus cDNA 5'	Ubiquitin
15	CB439150	688760 MARC 6BOV Bos taurus cDNA 3'	Signal transducer CD24 precursor
16	None	None	Unknown
17	CB444746	695948 MARC 6BOV Bos taurus cDNA 3'	Lactogen precursor (PL)
18	CB458763	717490 MARC 6BOV Bos taurus cDNA 3	MHC1 Ag
19	CD287708	1_C17.abd POE14_(Day_14_pregnant_ovine _endometrium) Ovis aries	Cytochrome oxidase
20	CB539079	777237 MARC 6BOV Bos taurus cDNA 3'	Unknown protein
21	CB533000	757497 MARC 6BOV Bos taurus cDNA 5'	Elongation factor 1-alpha 1
22	CB421980	595044 MARC 6BOV Bos taurus cDNA 3'	Unknown
23	CB226128	1RT28G05 Bos taurus Reticulum #1 library Bos taurus cDNA	Restin
24	None	None	Unknown
25	CB445242	696494 MARC 6BOV Bos taurus cDNA 5'	GTP binding protein

Table 5.4.B: Second batch of SSH contig alignment groups.

Contig	EST hit	Description	Tentative ID
1	64054 MARC	Cytochrome c oxidase subunit II	Cytochrome c oxidase subunit II
2	64054 MARC	Homologue to O-acetyltransferase	O-acetyltransferase
3	64054 MARC	None	Unknown
4	AJ690882	Sequence from clone RP1-12G14 on chromosome 6q24.1-25.2	Cytochrome b
5	64054 MARC	None	Unknown
6	971840 BARC 5BOV	Homo sapiens tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein
7	64054 MARC	None	Unknown
8	None	None	Unknown
9	10_A14.abd POE14_(	Homo sapiens ribosomal protein S16 (RPS16)	Ribosomal protein S16
10	None	None	Unknown
11	467760 MARC	Homo sapiens mRNA; cDNA DKFZp686N08224	Unknown
12	467760 MARC	Homo sapiens mRNA; cDNA DKFZp686N08224	Unknown
13	713880 MARC	Brown rat DNA sequence from SV40 infected cell line 14B	Unknown
14	722680 MARC	Ovis aries pulmonary surfactant-associated protein C	Surfactant protein C
15	972408 BARC	Homo sapiens cereblon	Cereblon

16	cattle|TC247089	Cattle homologue to RAD23B protein	RAD23B protein
17	CR456054	Homo sapiens RAD23 homolog B	RAD23B protein
18	fp876.z1 fp 73-75d	Bos taurus thymosin beta-10	Thymosin beta-10
19	CR552411	Mus musculus similar to ribosomal protein L30	Ribosomal protein L30
20	964338 MARC	Homo sapiens plastin 3 (T isoform) (PLS3)	Plastin 3
21	Oa_splbn_02P22_M	Ovis aries complete mitochondrial genome	Mitochondrial
22	None	None	Unknown
23	None	Ovis aries chromosome X centromeric satellite I DNA sequence	centromeric satellite I
24	None	None	Unknown
25	SP|Q06002|BFS1_B	Filensin (Beaded filament structural protein)	Filensin
26	Oa_splbn_02G06_M	Ovis musimon mitochondrial DNA for cytochrome b	Cytochrome b
27	Oa_splbn_03H15_M	Bos taurus thymosin beta 4 mRNA	Thymosin beta-4
28	Oa_splbn_09L08_M	Rattus norvegicus ribosomal protein L4	Ribosomal protein L4
29	726643 MARC	Diubiquitin	Diubiquitin
30	4100953 BARC	Destrin (Actin-depolymerizing factor)	Actin-depolymerizing factor
31	1RT28G0	Homo sapiens restin	Restin
32	1_I15.abd POE14_(	Ovis aries beta-2 microglobulin	Beta-2-microglobulin
33	None	None	Unknown

34	4092646 BARC	RTLF	Unknown
35	SP|P2382 1|RS7	Ribosomal protein S7 (S8).	Ribosomal protein S7
36	719610 MARC	None	Unknown
37	Oa_splbn_ 14L20_M	Ovis aries ribosomal protein S25 mRNA	Ribosomal protein S25
38	CR550390	Bos taurus cell division cycle 10 (CDC10)	Cell division
39	None	None	Unknown
40	None	Polymeric-immunoglobulin receptor precursor (Poly-Ig receptor) (PIGR)	Poly-Ig receptor
41	Oa_splbn_ 04P11	Bos taurus ubiquitin-S27a fusion protein	Ribosomal protein S27a
42	UMC-bend_0B0 1-015-f02	Human phosphotyrosine	Phosphotyrosine
43	CR552843	None	Unknown
44	Oa_splbn_ 04D12_M	Mus musculus ribosomal protein S7 (Rps7), mRNA	Ribosomal protein
45	Oa_splbn_ 10I03_M1	Ovis aries complete mitochondrial genome	Mitochondrial
46	cattle|AW 427705	cattle|AW427705	Unknown
47	Oa_splbn_ 08K17_M	Ovis aries complete mitochondrial genome	Mitochondrial
48	None	NADH-ubiquinone oxidoreductase	NADH-ubiquinone oxidoreductase
49	fp290.z2 fp 73-75d	Homo sapiens ribosomal protein L23a	Ribosomal protein 23a
50	None	None	Unknown
51	PIR|I3735	Epithelial microtubule-	Epithelial microtubule-

	6|I37356	associated	associated
52	W427705	>cattle|AW427705	Unknown
53	None	Cytochrome c oxidase subunit III [Bos taurus]	Cytochrome c oxidase subunit III
54	None	Cytochrome c oxidase subunit III [Bos taurus]	Cytochrome c oxidase subunit III
55	None	None	Unknown
56	None	None	Unknown
57	None	None	Unknown
58	UP|Q8U4 H9	O.aries centromerically located satellite DNA	Centromerically located satellite DNA
59	None	None	Unknown
60	619209 MARC	Mannose/glucose-binding lectin precursor	Mannose/glucose-binding lectin precursor
61	Oa_splbn_ 05C17_M	Ovis aries cytochrome c-oxidase subunit three (COIII)	Cytochrome c-oxidase subunit three (COIII)
62	Oa_splbn_ 13C21_M	Homo sapiens ribosomal protein L35 (RPL35),	Ribosomal protein L35
63	None	None	Unknown
64	fs485.z1 fs 103-	Sus scrofa ribosomal protein S23	Ribosomal protein S23
65	457377 MARC	Homo sapiens cytochrome b-245, beta polypeptide	Cytochrome b-245
66	CES01547	Diubiquitin	Diubiquitin
67	Oa_splbn_ 05L23_M	Ovis aries complete mitochondrial genome	Mitochondrial
68	152792 MARC	Homo sapiens CD24	CD24
69	Oa_splbn_ 07J07_M1	Ovis aries ferritin heavy-chain	Ferritin
70	SP|P0125 3|TYB4	Thymosin beta-4 (FX)	Thymosin beta-4

71	None	None	Unknown
72	961522 MARC	Homo sapiens solute carrier family 39 (zinc transporter),	Solute Carrier
73	OB142	Bos taurus thymosin beta 4	Thymosin beta-4
74	11_J11.abd POE14	Bos taurus ribosomal protein S12	Ribosomal protein S12
75	594389 MARC	Ovis aries pulmonary surfactant-associated protein A	Surfactant protein A

Table 5.4 lists the search results for each contig group. For each sequence a general database search both against the NCBI database and the TIGR cluster database was conducted in order to attempt to make a tentative identification of the gene from which the sequence originally came. These suggested identities are also listed in Table 5.4.

Microarray quality control

A list of all elements present on the microarray, and the information pertinent to each is presented in Appendix II, table 1. The red and green dyes showed no significant variability between them as assessed by the internal quality control used by the microarray analysis software. The red vs. green dye swaps are plotted against each other in Figure 5.3. In this figure the averaged and normalized values of each array element are plotted on each axis with the result of the replicate slide in which baseline cDNA was labelled with Cy3 (and experimental, post-LPS cDNA with Cy5) on the y axis (green/red) and the replicate in which the samples were labelled the other way around plotted on the x axis (red/green). The result is a figure illustrating the bias across each slide resulting from differential fluorescence readings due to dye type used. If no dye bias is present there will be no overall trend in the plotted points – and this is the case in figure 5.3 indicating that there was no dye bias inherent in the array results.

No slides showed significant within-slide variability was seen when assessed by the analysis software. Bovine and SSH clones were printed at random on the microarray in order to avoid any potential systematic bias across the slides. The analysis software used was able to sort the expression data based on which group of clones each element was derived from (bovine or SSH). This analysis found that bovine spots and SSH clones showed no significant differences in their tendency to hybridise

effectively. There were no systematic patterns of variable expression between SSH and bovine clones – i.e. as many showed up as down regulation from each group, and as many bovine as SSH clones were significantly changed in either segment.

Location of spot on the microarray slides appeared to play no part in expression level by visual inspection. Examples of hybridised arrays are shown in figure 5.4.

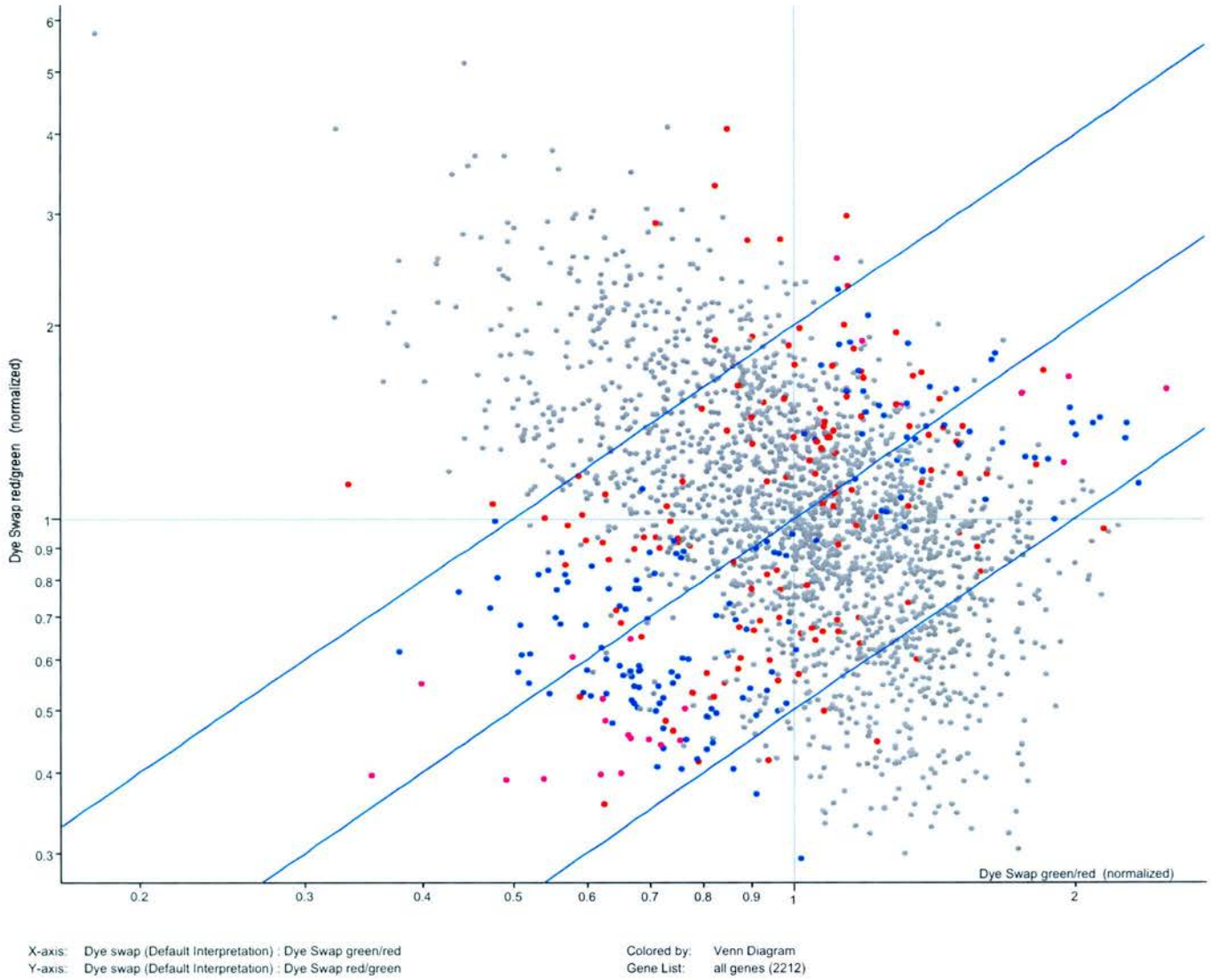


Figure 5.3: Scatter plot of each array element's normalised fluorescence value measured when the intercalating dyes (Cy3=green, Cy5=red) were exchanged in each sample. Each point on the graph represents an element on the array with all of the data obtained for that element in each of the two dye swap arrangements plotted against the other. This graph therefore represents all of the data collected from all of the array hybridisations, and is an indication of any systematic bias towards one dye or the other across the experiment. Red points: significantly altered in the direct segment, blue points: remote and purple: both. Grey spots did not show any significant variation in the standard analysis. The points are clustered around the central point as expected, and there is no evidence of dye-based bias.

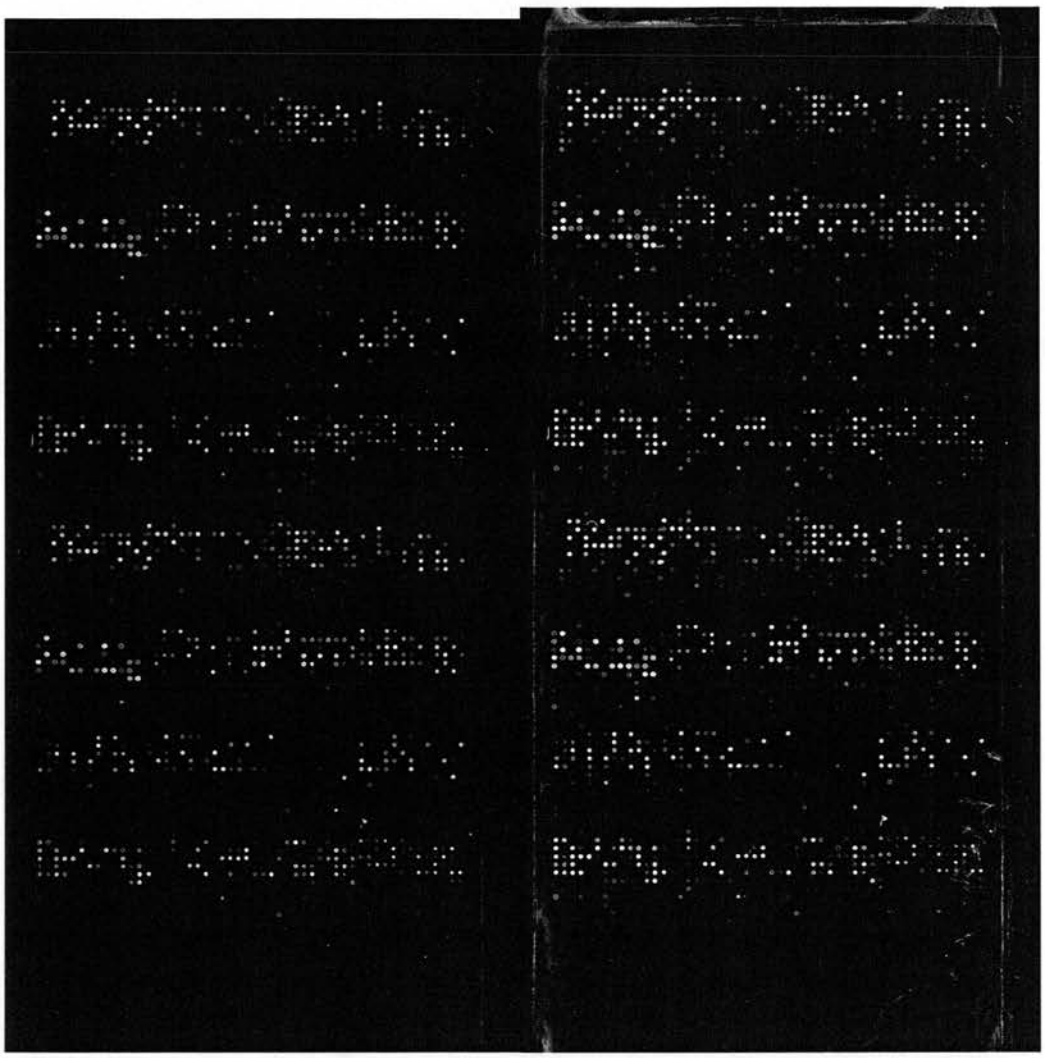


Figure 5.4.A: Scanned image of the ovine lung microarray following hybridisation to cDNA sample labelled with Cy3 dye. Each spot is clear and all but a very small proportion show uniform morphology. There is no evidence of systematic spatial bias in hybridisation efficiency across the slide. Visual inspection reveals no evidence of smeared elements, or extrinsic interference from contaminants. The Cy3 hybridisation gave similar results to the Cy5 hybridisation in terms of pattern of fluorescence and quality of image. Overall the array hybridised successfully and consistently, and was of appropriate quality for data acquisition

Figure 5.4.B: Scanned image of the ovine lung microarray following hybridisation to cDNA sample labelled with Cy5 dye. Each spot is clear and has uniform morphology. There is no evidence of systematic spatial bias in hybridisation efficiency across the slide. Visual inspection reveals no evidence of smeared elements, extrinsic interference from contaminants. The Cy5 hybridisation gave similar results to the Cy3 hybridisation in terms of pattern of fluorescence and quality of image. The image intensity overall is marginally higher than in the Cy3 array, though this would be corrected during the normalisation process.

Discussion

Identification of ovine homologs to bovine clones.

The MARC genomic cattle library had a considerable number of highly homologous clones with the ovine genome, as expected from such closely related species. Due to the bias inherent in the known and characterised ovine gene sequences there were disproportionate numbers of homologous clones representing MHC genes, housekeeping genes, immunoglobin genes and inflammatory mediator genes. These categories are over-represented due to the prevalence of immunology-based research in the sheep and as a result the set used on the microarray is suited to immune-inflammatory research purposes such as the investigation of LPS-induced inflammation.

The large set of housekeeping genes present within the bovine clones are also of functional importance in terms of the data analysis arising from the microarray investigation as they provide an alternate route to normalisation other than global strategies. The clones found using this technique represent commonly expressed and highly abundant mRNA species which complement the SSH strategy and allow for a robust and representative microarray to be constructed.

Suppressive subtractive hybridisation library

The subtractive suppression library technique has proved in the past to be an appropriate method of gene discovery in a specific and sensitive manner. The results from this experiment have shown that the technique is also applicable to the ovine lung, as it has isolated a number of sequences that are as yet unidentified within the sheep genome. Of the 238 sequences analysed, ten were discarded due to the sequence being empty, and a further two were eliminated as they represented stretches

of cloning vector only, this left 226 fully representative transcript sequences which fell naturally into 101 contig assemblies. Two of these contigs were identical complements of each other and can therefore be considered as one group leaving 100 unique transcript sequences, thus within the 238 clones there is approximately 2.4-fold redundancy.

A number of the sequences analysed showed homology with known sheep genes, with greater then 70% of the remainder showing strong homology with clones derived from other ovine or bovine EST libraries. Approximately 25% of the sequences analysed showed no homology with any EST or annotated gene sequences – and this could be due to a number of factors. It is possible that these sequences represent "junk" DNA unrepresentative of any real expressed transcript or are a result of some other artefact of the experimental process. The other possibility is that these sequences are ovine specific and have thus not been detected in other species (particularly bovine) library project, or that they are highly lung specific and expressed at low level, and again never been detected previously.

The majority of the analysed sequences showed strong homologies with known bovine EST sequences, mostly from the large scale MARC library bovine genome sequencing project and this is a strong indication that they are "genuine" transcripts representing – for the main part – uncharacterised gene products in the sheep. The majority of the contig groups from the sequenced products contained one or two sequences, indicating that the selection was still far from saturated and that there are thus a number more sequences to be found.

1,200 SSH clones were PCR amplified and the products used in the manufacture of a novel microarray. Were the percentage coverage within the SSH clones to remain constant over this number of inserts it would be expected that they would represent

between 400 and 500 unique transcripts. Even if this number is not achieved it seems highly probable that fifty or more novel ovine ESTs will be represented. There are currently approximately 4000 ovine sequences in the NCBI gene database, so this library represents anywhere from five to ten percent of the known ovine genome and is therefore a significant resource in terms of gene discovery. Further characterisation of the sequences obtained by this method also represents a new avenue into the continued investigation of both the ovine transcriptome and the molecular nature of the ovine lung.

Microarray Performance

The new array performed well overall as the images acquired looked clean, lacking significant background interference and resolving clear, uniform spotted elements. There appeared to be little if any smearing of the spotted cDNA and the grids were uniformly distributed and positioned as intended – indicating that the mechanical printing was a success. The quantified results appeared to be due to biological change and not systematic bias within array as the red/green balance was even, and there was no discernable spatial bias across any of the slides. The cDNA spots showed equal effectiveness as probes when derived from either the bovine library clones or the subtracted suppressed hybridisation generated library clones.

Overall the global normalisation approach used assigned the majority of housekeeping and other elements that would not be expected to change to the 1-fold unchanged region, and did not find them to be significantly altered. However actin, often used as a housekeeping gene, was found to be significantly down regulated along with a number of related cell structure genes, including actin binding and regulating elements. This change is consistent with findings by previous studies (Glare et al. 2002) that suggest alterations in beta-actin during lung disease and challenge and

which call into serious question the use of beta-actin as a reference or housekeeping gene in the lung. These data indicate that the array worked as expected and strongly suggest that the overall biological results from this platform are viable and genuine.

Meta-analysis of the results obtained from the array show that approximately 5% of the elements present changed significantly in the directly challenged segment. This figure is lower than has been reported in previous comparable studies, however this was likely to be due to the nature of the statistical tests and low biological number of replicates (n=5) which make statistical significance less likely. Similarly there were approximately 5% of the elements altered significantly in the contra-lateral remote segment – this result may be more as expected due to the subtlety of the remote response, consequent to which the numbers of affected genes would perhaps be expected to be lower than in a grossly affected tissue population.

Conclusions

The combination of SSH library construction and BLAST identification of homologous genes is a rapid and efficient means of gene discovery of direct application to the generation of a novel microarray. The gene sequences thus discovered cover those products expressed at high levels and commonly throughout the sheep, as well as rare transcripts specific to or differentially expressed in the ovine lung. The microarray thus produced is therefore suited to a wide range of investigative areas within ovine pulmonary biology and represents a considerable resource in that regard.

Chapter 6 Microarray analysis

Aims and objectives

- To determine the nature and extent of contra lateral remote lung responses following local lung instillation of LPS
- To compare and contrast such responses to those occurring in response to direct challenge with LPS

Introduction

The inflammatory and immune response of the lung to LPS has been shown to be multi-faceted with the characterised aspects of the response involving large numbers of disparate genes, gene products and pathways (Prince et al. 2001; Yamanaka et al. 2001; Moore & Standiford 1998). It is probable that such characterised elements represent a fraction of the total change in gene expression involved in such a response. Indeed the diversity of genes involved in response to LPS has been previously highlighted in the context of murine lung in a whole lung *in vivo* study (Katsuma et al. 2001) and human neutrophils (Malcolm et al. 2003), endothelial cells (Zhao et al. 2001) and epithelial cells (Yamanaka et al. 2001; Ichikawa et al. 2000).

To emphasise the extent of this the study by Malcolm et al demonstrated significant regulation of cytoskeletal genes and genes involved with cell cycle control. These genes are not considered a usual part of the innate inflammatory response. These studies have shown that the response to LPS is not confined to genes conventionally held to be a part of the inflammatory or immune cascades. The contention held here is that such diversity will also be reflected in the ovine lung response to LPS.

The power of the microarray as a molecular analysis platform is its ability to simultaneously and quantitatively determine the expression levels of many genes in a given RNA population (Duggan et al. 1999). This has been likened to providing a 'snapshot' of the transcriptional activity within the sample (Francois et al. 2003; Neumann & Galvez 2002). Microarrays may therefore indicate the nature and extent of genetic networks and offer particular advantage over more conventional molecular techniques such as PCR or *in situ* hybridisation in providing insight into the overall complexity of gene expression responses. These qualities render microarrays suitable for detecting subtle changes in transcription between closely related samples in a quantitative and robust manner. The assertion here is that a microarray platform is ideally suited to detecting the nature and extent of hypothesised changes in gene expression in ovine lung segments remote to the site of local LPS challenge.

An expanding literature base surrounds the use of microarray technology in defining and dissecting lung responses. Indeed previous studies in the lung have investigated changes in a number of cell and tissue types including neutrophils (Malcolm et al. 2003), endothelial cells (Zhao et al. 2001; Neilsen, Zimmerman, & McIntyre 2001) and epithelial cells (Ichikawa et al. 2000) *in vitro*, as well as whole lung, endothelium and epithelial brushings *in vivo* in samples derived from mice (Katsuma et al. 2001) rats (Wiethoff et al. 2003; Liu et al. 2003; Hoshikawa et al. 2003) and humans(Wolfgang et al. 2004; Laprise et al. 2004)..

One significant caveat in relation to the interpretation and implied value of microarray data concerns the analysis of often very large data sets. As outlined by Stark et al 2003 devising methods that can extract useful information about biological function from the vast amounts of data being generated by such technologies is a major challenge. Conclusions drawn on the basis of current techniques are largely

descriptive, without necessarily leading to deeper insights about how gene interactions control cellular function. It is with this limitation in mind that the objectives of the present chapter have been defined.

Results

Remote segment gene expression

Examples of the scanned microarrays in false colour images are shown in figure 6.1. Figures 6.1a (Cy3) and b (Cy5) show arrays from the remote lung segments and 6.1c (Cy3) and d (Cy5) from the directly challenged segments. The remaining images are shown in Appendix III along with all raw data in Appendix IV. Figure 6.1.B shows an example array slide in which the overall fluorescence intensity is higher on the left than the right, probably due to a slight inaccuracy in the positioning f the slide in the loading cartridge. This type of systematic bias across an array slide is accounted for during the data capture and analysis processes. During data capture the image is analysed in terms of each array spot individually, with total fluorescence information for the spot, its immediate neighbours, its immediate block and the overall fluorescence intensity of the array. Each spot is then normalized within and without its near-neighbour spots and blocks, resulting in a spot value that is corrected for the systematic bias across the slide as well as any local deviant areas or localised artefactual anomalies.

The normalized results from the microarray analysis of gene expression in the remote segment indicated a number of significantly regulated genes following LPS exposure. The results of the overall changes in the remote segment are shown as a scatter diagram in figure 6.2. The points plotted in figure 6.2 are coloured by significance of expression change with increasing red colour intensity indicating more significant up

regulation of the element and increasing green colour intensity indicating more significant down regulation of the element. The majority of the array elements were found to be at or around the 1-fold mark indicating no change from baseline following LPS challenge. Overall there were fewer elements that were up than down regulated.

A number of control elements were included in the array design. These broadly comprised three distinct subgroups – housekeeping gene controls, negative control genes and negative spot controls. Negative control spots comprised spotted SSC (i.e. a totally non-DNA spot). These spots allowed for assessment and control of non-DNA binding to the slide substrate itself, and as such comprised the 'absolute baseline' control. The analysis software used integrally analysed fluorescence from these spots and adjusted all subsequently analysed spots based on the data acquired from them.

Negative control genes comprised genetic sequences from non-mammalian species (i.e. plant, bacterial and synthetic 'nonsense' sequences) and controlled for non-specific DNA-DNA binding. These control sequences were analysed prior to printing to ensure no sequence homology was found with any experimental genes, they therefore provided a means of subtracting the non-specific binding on the array due to DNA-DNA interactions that did not depend on homology for their strength. The housekeeping gene controls used were those previously published as maintenance genes or those often used in experimental settings. Those used included ATPase, β-actin and GAPDH. None of these showed significant deviation from baseline.

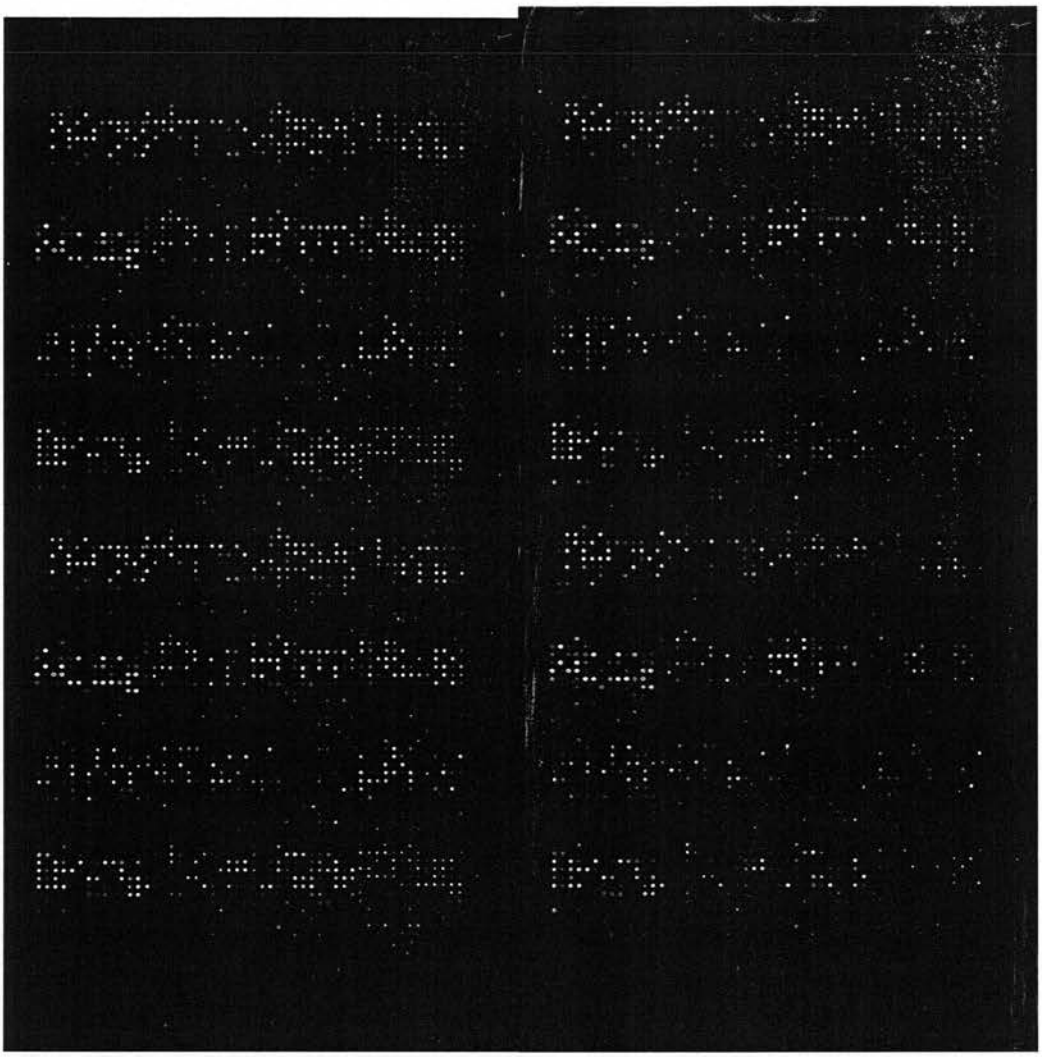


Figure 6.1.A: Scanned image of the ovine lung microarray following hybridisation to a cDNA sample taken from the contra lateral lung segment at baseline labelled with Cy3 dye. Each spot is clear and all but a very small proportion show uniform morphology. There is no evidence of systematic spatial bias in hybridisation efficiency across the slide. Visual inspection reveals no evidence of smeared elements or extrinsic interference from contaminants. This sample is taken from the same sheep as the image from the Cy5 sample alongside.

Figure 6.1.B: Scanned image of the ovine lung microarray following hybridisation to a cDNA sample taken from the contra lateral lung segment at 6 hours post-LPS labelled with Cy5 dye. Each spot is clear and has all but a very small proportion show uniform morphology. There is some evidence of systematic low fluorescence bias in along the right hand side of the slide. There is no evidence of smeared elements or extrinsic interference from contaminants.

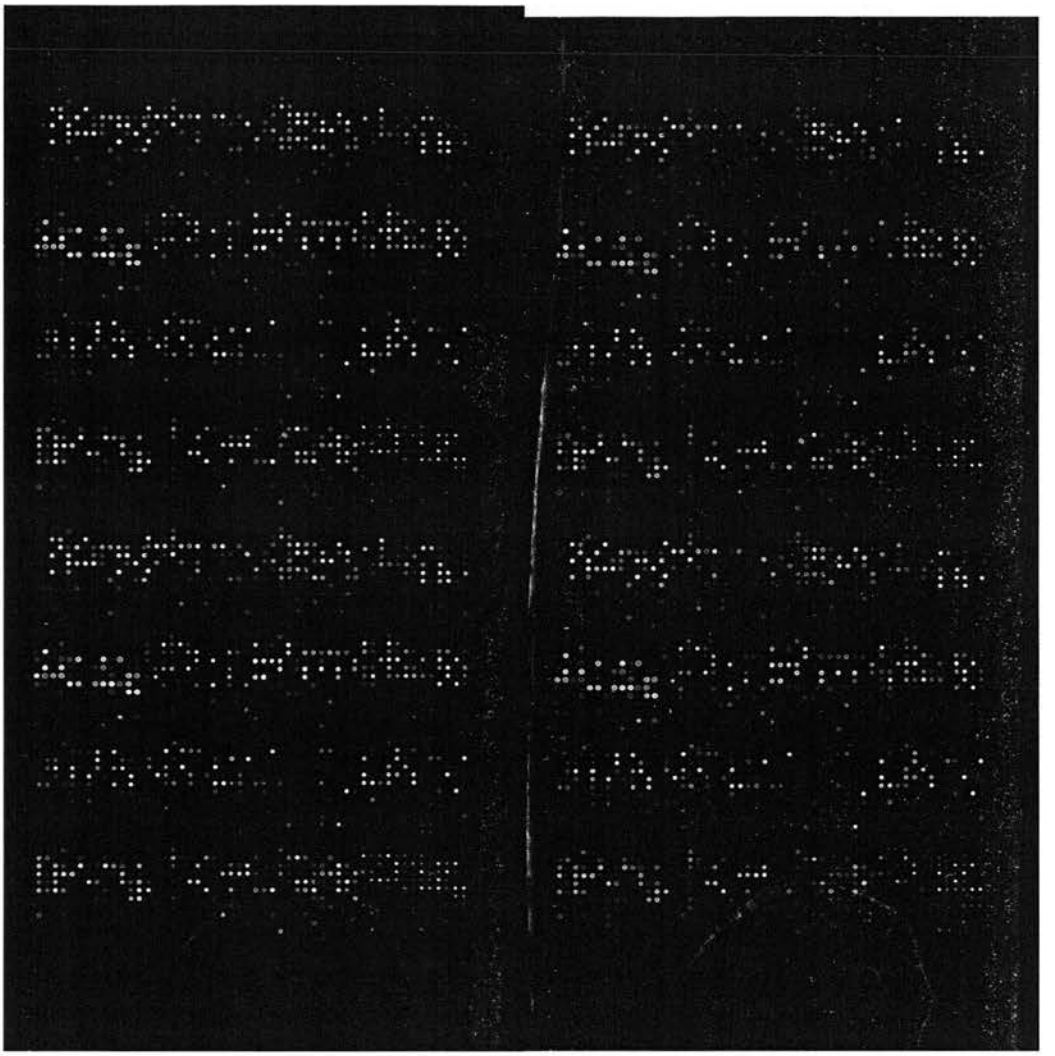


Figure 6.1.C: Scanned image of the ovine lung microarray following hybridisation to a cDNA sample taken from challenged lung segment at baseline labelled with Cy3 dye. Each spot is clear and has all but a very small proportion show uniform morphology. There is no evidence of systematic spatial bias in hybridisation efficiency across the slide. Visual inspection reveals no evidence of smeared elements or extrinsic interference from contaminants. This sample is taken from the same sheep as the image from the Cy5 sample alongside.

Figure 6.1.D: Scanned image of the ovine lung microarray following hybridisation to a cDNA sample taken from the challenged lung segment at 6 hours post-LPS labelled with Cy5 dye. Each spot is clear and has all but a very small proportion show uniform morphology. There is some evidence of systematic low fluorescence bias in along the right hand side of the slide. There is no evidence of smeared elements or extrinsic interference from contaminants.

In order to assess the numbers of elements which showed significant changes in expression in the remote segment genes were filtered to show those which passed a student t-test with a score at $p<0.05$ ($n=8$) after a false positive reporting correction. These data are illustrated in the scatter graph shown in figure 6.3. 170 elements were found to have changed significantly. False positive correction was applied in order to account for the relatively high number of chance positives acquired even at a 95% significance level due to the high sample number. Prior to correction 382 elements were found to have changed significantly.

One element showed greater than 2-fold significant up regulation and was assigned to the 'high up' category (table 6.1). Fifteen elements showed greater than 2-fold significant down regulation and were assigned to the 'high down' category (table 6.2). Fifty-five elements showed between 1 and 2-fold significant up regulation and were assigned to the 'low up' category (table 6.3). Ninety-nine elements showed between 1 and 2-fold significant down regulation and were assigned to the 'low down' category (table 6.4).

Where possible each element showing significant regulation was assigned to a predicted functional classification. The number of elements in each predicted functional classification and expression category (high/low, up/down) are displayed as a 3D bar chart in figure 6.4.

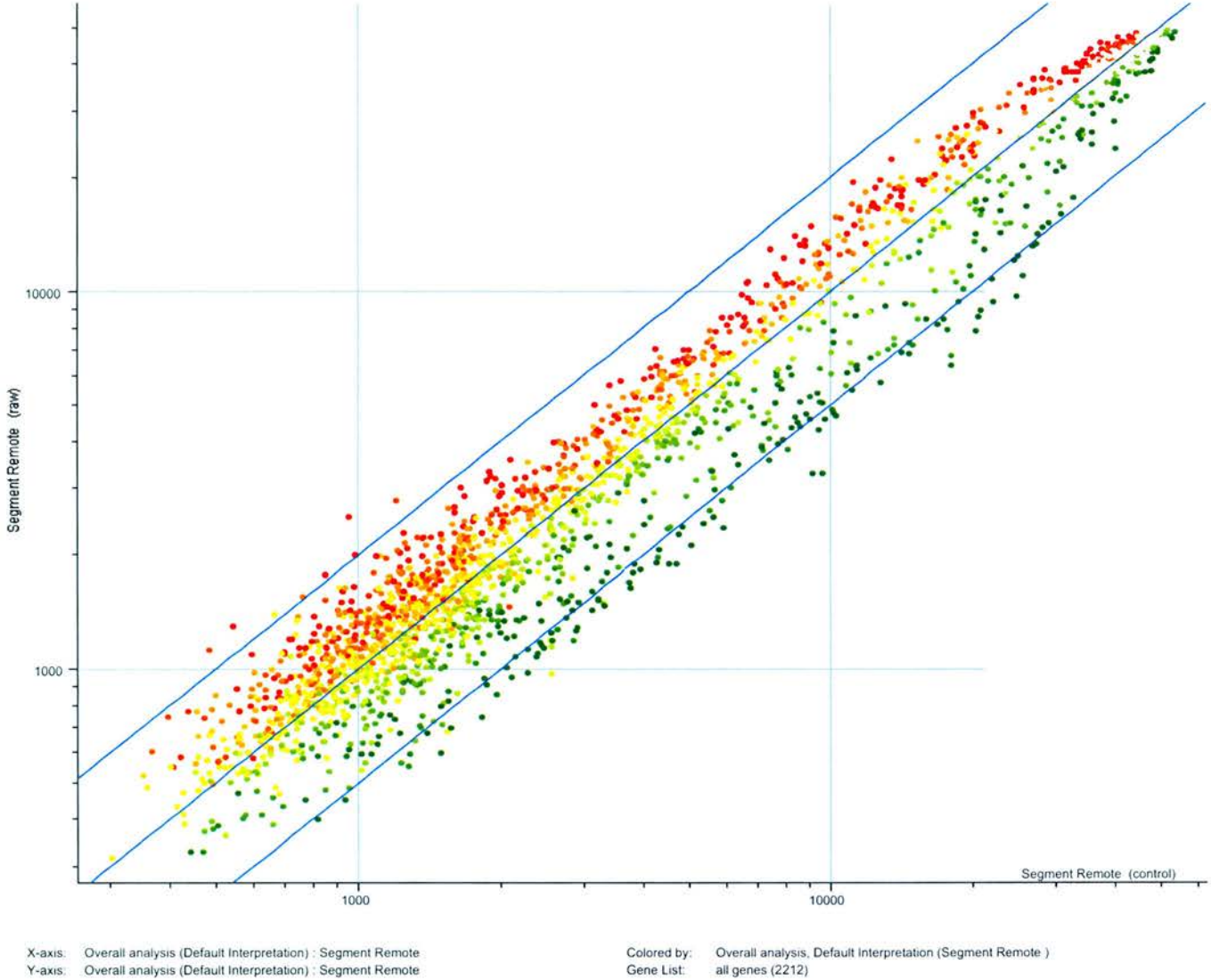


Figure 6.2: Scatter plot of each microarray element's normalised fluorescence intensity at baseline (x axis) vs. 6 hours post-LPS challenge (y axis) in the contra lateral lung. Blue lines (from left to right) represent approximately 2, 1, and 0.5-fold change respectively. Each point on the graph represents an element on the array, and is coloured according to significance, i.e. the more intensely red the point, the higher the significance of up regulation is, whilst the more intense the green colour, the higher the significance of down regulation is. Elements that are have a weak or intermediate colour show low levels of statistical significance. Clearly the majority of elements are not significantly altered in terms of their expression levels, and there are a number whose mean expression alters considerably but not to significant levels.

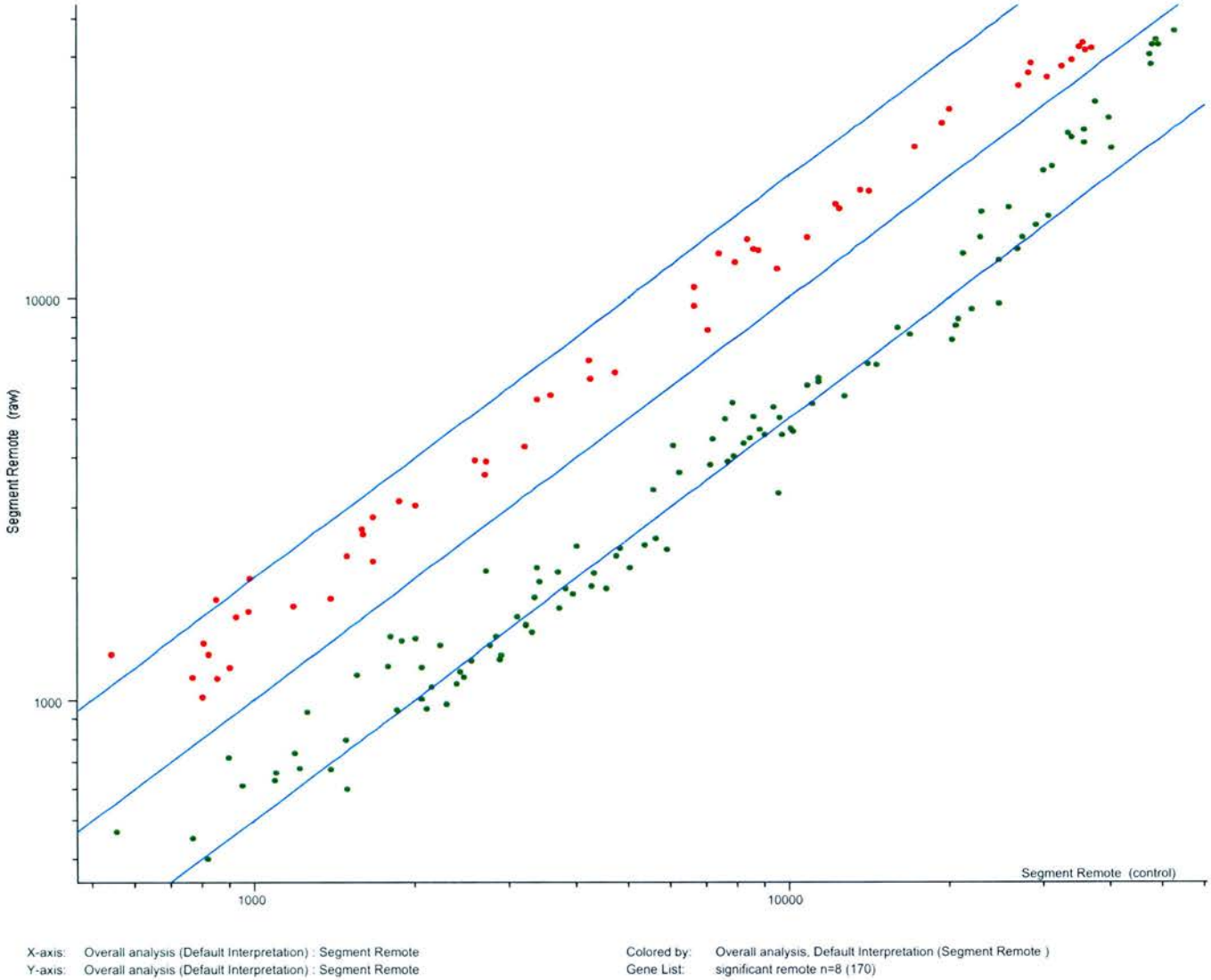


Figure 6.3: Scatter plot of the microarray elements (baseline (x axis) vs. 6 hours post-LPS challenge (y axis)) that showed significantly altered expression in the contra lateral lung. Blue lines (from left to right) represent approximately 2, 1, and 0.5-fold change respectively. Each point on the graph represents an element on the array, with red points representing up regulated elements and green points representing down regulated ones. The majority of the significantly changed elements fall between the outer two blue lines, indicating less than 2-fold change in either direction. The spread of genes within the range is very even with regard to intensity of expression, those genes closest to the origin of the graph were expressed at the lowest levels. This indicates successful data normalisation.

Table 6.1: Details of the gene that was classified as highly (>2 fold) up regulated in the remote segment at 6 hours post-LPS treatment. The fold change represents the mean value of the normalised fluorescence intensities of the labelled cDNA samples derived from the RNA taken from the post-LPS instillation (6 hours) brushed epithelial cells divided by the normalised fluorescence intensities of the labelled cDNA samples derived from the RNA taken from the baseline (0 hours) brushed epithelial cells from the sheep analysed (n=8). Functional classification was based on homology analysis of the probe sequence combined with literature searches.

Gene name	Fold change	Function
Em4b	2.152	Immune/Inflammatory

Table 6.2: Details of the genes that were classified as highly (<0.5 fold) down regulated in the remote segment at 6 hours post-LPS treatment. The derivation of the fold change and assigned functional classification was as described in Table 6.1.

Gene name	Fold change	Function
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	0.454	Cell cycle
Cell division	0.484	Cell cycle
Thymosin beta-10	0.392	Cell structure
Filensin	0.493	Cell structure
Centromeric satellite I	0.457	Centromere
Surfactant protein C	0.48	Membrane
Immunoglobulin mu heavy chain variable region	0.496	Membrane
Cytochrome b (Swissprot)	0.475	Metabolic
Cytochrome b (Swissprot)	0.479	Metabolic
Alpha-S1-casein	0.477	Milk
Ribosomal protein S27a	0.481	Ribosomal
Unidentified SSH clone insert	0.462	Unidentified
Unidentified SSH clone insert	0.473	Unidentified
Phospho-tyrosine	0.454	Transcription Factor
RAD23B protein	0.467	Unknown

Table 6.3: Details of the genes that were classified as showing low (<2 fold) up regulation in the remote segment at 6 hours post-LPS treatment. The derivation of the fold change and assigned functional classification was as described in Table 6.1.

Gene name	Fold change	Function
Bcl2-associated protein Bax Bax	1.496	Cell cycle
Cathepsin D CTSD	1.585	Cell cycle
Hyal2 protein Hyal2	1.39	Cell structure
Growth hormone	1.616	Growth hormone
Growth hormone	1.647	Growth hormone
Complement component C3 C	1.433	Immune/Inflammatory
Prostaglandin endoperoxidase	1.872	Immune/Inflammatory
Interferon-gamma-inducible protein-10	1.923	Immune/Inflammatory
Kunitz domain protein	1.818	Kunitz
MHC class I protein	1.271	Membrane
Immunoglobulin mu heavy chain variable region	1.413	Membrane
MHC class I protein	1.444	Membrane
MHC OVAR-DQ-ALPHA1	1.617	Membrane
CD3 gamma subunit	1.749	Membrane
Cystic fibrosis transmembrane conductance regulator CFTR gene, intron 1	1.8	Membrane
Beta-casein	1.853	Membrane
T-cell receptor beta-chain	1.907	Membrane
Cytochrome c oxidase subunit III	1.262	Metabolic
Cytochrome b	1.357	Metabolic
Lipoprotein lipase	1.459	Metabolic
Muscle-type carnitine palmitoyltransferase I, CPTIB gene	1.474	Metabolic
Cytochrome c-oxidase subunit three COIII	1.497	Metabolic

gene, complete cds and tRNA-Gly		
Mitochondrial cytochrome b	1.55	Metabolic
Cytochrome b	1.631	Metabolic
Cytochrome P450 steroid 17alpha-hydroxylase /17,20 lyase CYP17	1.724	Metabolic
Cytochrome b	1.792	Metabolic
Mitochondrial	1.171	Mitochondrial
Mitochondrial	1.18	Mitochondrial
Mitochondrial	1.206	Mitochondrial
Mitochondrial	1.342	Mitochondrial
Mitochondrion	1.45	Mitochondrial
Diubiquitin	1.55	Mitochondrial
Mitochondrion	1.685	Mitochondrial
Mitochondrion	1.72	Mitochondrial
Mitochondrion	1.872	Mitochondrial
Ribosomal protein 23a	1.191	Ribosomal
Ribosomal protein 23a	1.228	Ribosomal
Ribosomal protein 23a	1.264	Ribosomal
MARC 9438-9439:991426302:1 Sheep WBCOvis aries STS genomic, sequence tagged site	1.403	Ribosomal
Ribosomal protein S25	1.438	Ribosomal
BI094681 et3-19 Sheep Lambda Zap II Library Ovis aries cDNA clone et3-19 5'	1.469	Ribosomal
Ribosomal protein S16	1.503	Ribosomal
Ribosomal protein S25	1.553	Ribosomal
Ribosomal protein	1.579	Ribosomal
AF354168 Ovis aries chromosome 18clone 229G11; 359E3, WORKING DRAFT SEQUENCE, 30 ordered pieces	1.527	Transcription factor

AF354168 Ovis aries chromosome 18clone 229G11; 359E3, WORKING DRAFT SEQUENCE, 30 ordered pieces	1.634	Transcription factor
Thyroid hormone receptor alpha1	1.814	Transcription factor
Unidentified SSH clone insert	1.36	Unidentified
Unidentified SSH clone insert	1.41	Unidentified
Unidentified SSH clone insert	1.436	Unidentified
Unidentified SSH clone insert	1.498	Unidentified
Unidentified SSH clone insert	1.603	Unidentified
Similar to TPRC	1.781	Unknown
SEG_F325416S	1.828	Unknown
Unknown	1.555	Unknown

Table 6.4: Details of the genes that were classified as showing low (>0.5 fold) down regulation in the remote segment at 6 hours post-LPS treatment. The derivation of the fold change and assigned functional classification was as described in Table 6.1.

Gene name	Fold change	Function
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	0.536	Cell cycle
Plastin 3	0.507	Cell structure
Actin-depolymerizing factor	0.509	Cell structure
Beta actin	0.519	Cell structure
Thymosin beta-4	0.526	Cell structure
Thymosin beta-10	0.53	Cell structure
Epithelial microtubule-associated	0.583	Cell structure
Human beta-actin control	0.738	Cell structure
Thymosin beta-4	0.768	Cell structure
Thymosin beta-4	0.776	Cell structure
Thymosin beta-4	0.792	Cell structure
Centromeric satellite I	0.566	Centromere
Centromerically located satellite DNA	0.666	Centromere
Centromerically located satellite DNA	0.714	Centromere
Prostaglandin D2 synthase	0.594	Immune/Inflammatory
Osteopontin	0.667	Immune/Inflammatory
COX	0.87	Immune/Inflammatory
Decorin	0.534	Membrane
Immunoglobulin mu heavy chain variable region clone VRA17	0.561	Membrane
Surfactant protein C	0.569	Membrane
Solute Carrier	0.613	Membrane
Surfactant protein A	0.616	Membrane

CD24	0.623	Membrane
Decorin	0.701	Membrane
6-pgdh mRNA for 6-phosphogluconate dehydrogenase	0.509	Metabolic
Cytochrome b	0.513	Metabolic
Cytochrome b	0.529	Metabolic
Cytochrome b	0.531	Metabolic
Mannose/glucose-binding lectin precursor	0.531	Metabolic
Cytochrome b	0.535	Metabolic
Cytochrome b	0.553	Metabolic
Cytochrome b-245	0.557	Metabolic
Ferritin	0.593	Metabolic
Cytochrome b	0.595	Metabolic
Acetyl-CoA carboxylase alpha, 5' region	0.604	Metabolic
Cytochrome b	0.64	Metabolic
Cytochrome b	0.656	Metabolic
Cytochrome b	0.657	Metabolic
Cytochrome b	0.675	Metabolic
Cytochrome b	0.716	Metabolic
6-pgdh mRNA for 6-phosphogluconate dehydrogenase	0.751	Metabolic
Cytochrome c oxidase subunit III	0.873	Metabolic
Cytochrome b	0.915	Metabolic
Diubiquitin	0.56	Mitochondrial
Mitochondrion	0.587	Mitochondrial
Mitochondrial	0.856	Mitochondrial
Mitochondrial	0.877	Mitochondrial

Mitochondrial	0.904	Mitochondrial
Ribosomal protein S7	0.513	Ribosomal
Ribosomal protein S16	0.52	Ribosomal
Ribosomal protein L35	0.524	Ribosomal
Ribosomal protein S25	0.551	Ribosomal
Ribosomal protein S12	0.563	Ribosomal
Ribosomal protein S27a	0.566	Ribosomal
Ribosomal protein	0.573	Ribosomal
Ribosomal protein S25	0.583	Ribosomal
Ribosomal protein S25	0.617	Ribosomal
Ribosomal protein S12	0.626	Ribosomal
Ribosomal protein S23	0.642	Ribosomal
Ribosomal protein L32	0.651	Ribosomal
Ribosomal protein S16	0.764	Ribosomal
Transcription factor Ets-2	0.539	Transcription factor
Unidentified SSH clone insert	0.501	Unidentified
Unidentified SSH clone insert	0.51	Unidentified
Unidentified SSH clone insert	0.521	Unidentified
Unidentified SSH clone insert	0.522	Unidentified
Unidentified SSH clone insert	0.523	Unidentified
Unidentified SSH clone insert	0.525	Unidentified
Unidentified SSH clone insert	0.529	Unidentified
Unidentified SSH clone insert	0.533	Unidentified
Unidentified SSH clone insert	0.533	Unidentified
Unidentified SSH clone insert	0.538	Unidentified
Unidentified SSH clone insert	0.559	Unidentified
Unidentified SSH clone insert	0.573	Unidentified

Unidentified SSH clone insert	0.576	Unidentified
Unidentified SSH clone insert	0.578	Unidentified
Unidentified SSH clone insert	0.579	Unidentified
Unidentified SSH clone insert	0.58	Unidentified
Unidentified SSH clone insert	0.581	Unidentified
Unidentified SSH clone insert	0.582	Unidentified
Unidentified SSH clone insert	0.584	Unidentified
Unidentified SSH clone insert	0.59	Unidentified
Unidentified SSH clone insert	0.597	Unidentified
Unidentified SSH clone insert	0.617	Unidentified
Unidentified SSH clone insert	0.631	Unidentified
Unidentified SSH clone insert	0.645	Unidentified
Unidentified SSH clone insert	0.647	Unidentified
Unidentified SSH clone insert	0.65	Unidentified
Unidentified SSH clone insert	0.659	Unidentified
Unidentified SSH clone insert	0.663	Unidentified
Unidentified SSH clone insert	0.738	Unidentified
Unidentified SSH clone insert	0.764	Unidentified
Unidentified SSH clone insert	0.776	Unidentified
O-acetyltransferase	0.509	Unknown
Cereblon	0.522	Unknown
O-acetyltransferase	0.549	Unknown
O-acetyltransferase	0.553	Unknown
RAD23B protein	0.608	Unknown
Unknown	0.632	Unknown

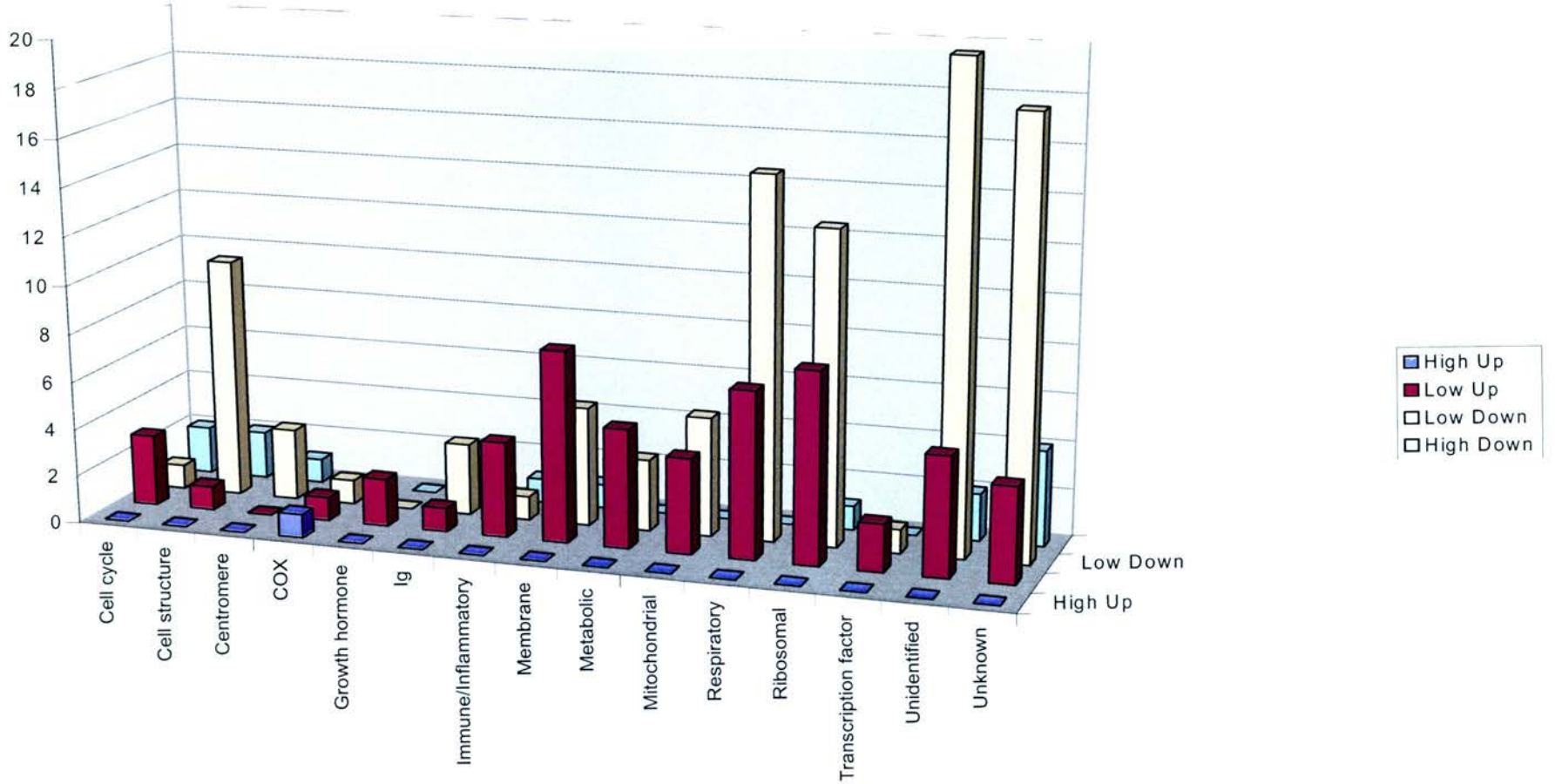


Figure 6.4: 3D bar chart displaying the number of elements showing significant regulation in the contra lateral lung segment 6 hours after local LPS challenge. The elements were grouped according to function (x axis) and also according to expression change (z axis). The number of elements represented in each of the resultant groups was then plotted on the y axis. The height of each column therefore represents the number of elements in that function/expression group. The highest peaks are those representing the 'low down' expression level for unidentified and unknown function elements. Of the identified elements cell structure (low down), respiratory and ribosomal (low up and down) and membrane (low up) are the most prominent peaks. Growth hormones were all up regulated at low levels, whilst centromere molecules were all down regulated.

Direct segment gene expression

The normalized results from the microarray analysis of gene expression in the direct segment indicated a number of significantly regulated genes following LPS exposure. The results of the overall changes in the direct segment are shown as a scatter diagram in figure 6.5. As for figure 6.1 the colouring of points reflects the intensity of expression. The majority of the array elements were found to be at or around the 1-fold mark indicating no change from baseline following LPS challenge. Overall there were very similar numbers of elements that were up and down regulated.

In order to assess the numbers of elements which showed significant changes in expression in the direct segment genes were filtered to show those which passed a student t-test with a score at $p<0.05$ ($n=5$) without false positive reporting correction. These data are illustrated in the scatter graph shown in figure 6.6. A total of 153 elements were found to have changed significantly.

Two elements showed greater than 2-fold significant up regulation and were assigned to the 'high up' category (table 6.5). Nine elements showed greater than 2-fold significant down regulation and were assigned to the 'high down' category (table 6.6). Seventy elements showed between 1 and 2-fold significant up regulation and were assigned to the 'low up' category (table 6.7). Seventy-two elements showed between 1 and 2-fold significant down regulation and were assigned to the 'low down' category (table 6.8).

Each element showing significant regulation was assigned to a functional classification. The functional classification numbers in each category (high/low, up/down) are displayed as a 3D bar chart in figure 6.7.

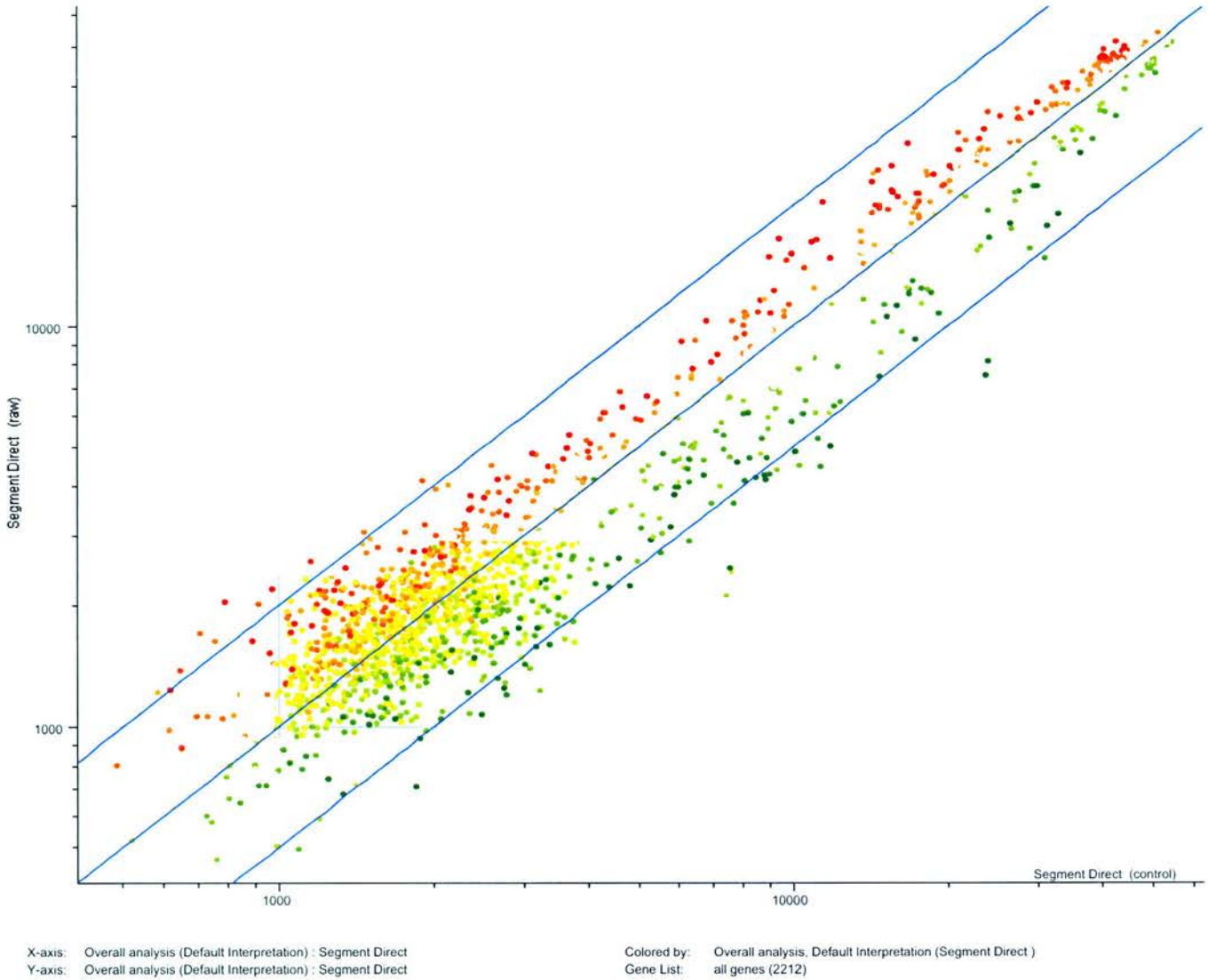


Figure 6.5: Scatter plot of each microarray element's normalised fluorescence intensity at baseline (x axis) vs. 6 hours post-LPS challenge (y axis) in the challenged segment. Blue lines (from left to right) represent approximately 2, 1, and 0.5-fold change respectively. Each point on the graph represents an element on the array, and is coloured according to significance, i.e. the more intensely red the point, the higher the significance of up regulation is, whilst the more intense the green colour, the higher the significance of down regulation is. Elements that are have a weak or intermediate colour show low levels of statistical significance. The number of significantly expressed genes is lower than in the contra lateral segment, and the spread of expression changes is greater with more bias towards up regulation.

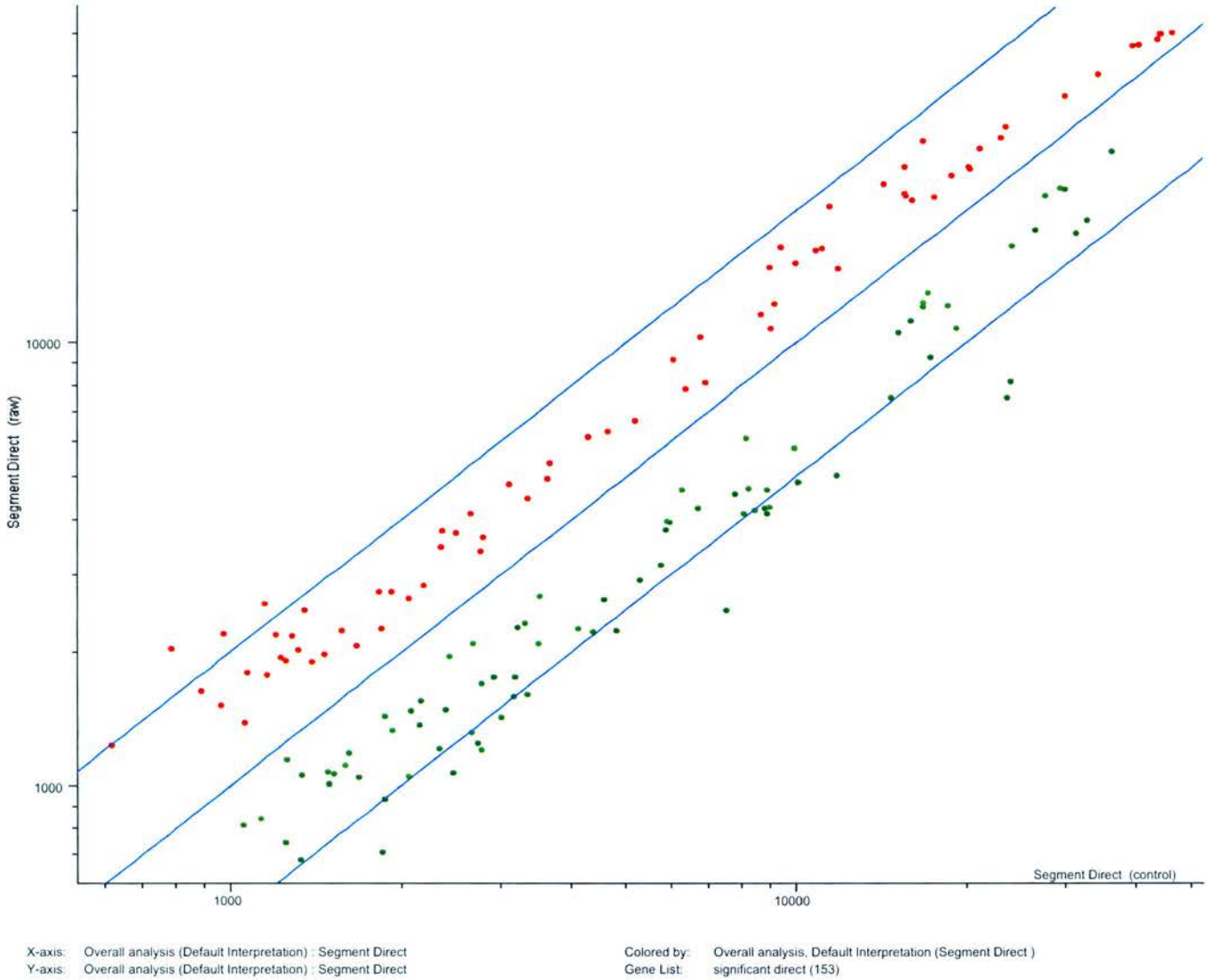


Figure 6.6: Scatter plot of the microarray elements (baseline (x axis) vs. 6 hours post-LPS challenge (y axis)) that showed significantly altered expression in the challenged lung segment. Blue lines (from left to right) represent approximately 2, 1, and 0.5-fold change respectively. Each point on the graph represents an element on the array, with red points representing up regulated elements and green points representing down regulated ones. The majority of the significantly changed elements fall between the outer two blue lines, indicating less than 2-fold change in either direction. The spread of genes within the range is similar to that seen in the remote segment. The points on this chart show less down regulated genes than in the remote segment, and a slight bias towards low expression elements.

Table 6.5: Details of the genes that were classified as highly (>2 fold) up regulated in the directly challenged segment at 6 hours post-LPS treatment. The fold change represents the mean value of the normalised fluorescence intensity of the labelled cDNA sample derived from the RNA taken from the post-LPS instillation (6 hours) brushed epithelial cells divided by the normalised fluorescence intensity of the labelled cDNA sample derived from the RNA taken from the baseline (0 hours) brushed epithelial cells from the sheep analysed (n=5). Functional classification was based on homology analysis of the probe sequence combined with literature searches and/or protein translation with predicted functional analysis.

Gene name	Fold change	Function
Cytochrome P-450 17-alpha-hydroxylase	2.526	Metabolic
Unidentified SSH clone insert	2.064	Unidentified

Table 6.6: Details of the genes that were classified as highly (<0.5 fold) down regulated in the directly challenged segment at 6 hours post-LPS treatment. The derivation of the fold change and assigned functional classification was as described in Table 6.5.

Gene name	Fold change	Function
Thymosin beta-10	0.344	Cell Structure
centromeric satellite I	0.46	Centromere
Endothelial nitric oxide synthase 23B	0.491	Immune/Inflammatory
TIMP-1=metalloproteinase tissue inhibitor	0.476	Immune/Inflammatory
Unidentified SSH clone insert	0.498	Unidentified
Unidentified SSH clone insert	0.48	Unidentified
Unidentified SSH clone insert	0.464	Unidentified
RAD23B protein	0.468	Unknown
Phototyrosine	0.41	Unknown
Thymosin beta-10	0.344	Cell Structure
centromeric satellite I	0.46	Centromere
Endothelial nitric oxide synthase 23B	0.491	Immune/Inflammatory

Table 6.7: Details of the genes that were classified as weakly (<2 fold) up regulated in the directly challenged segment at 6 hours post-LPS treatment. The derivation of the fold change and assigned functional classification was as described in Table 6.5.

Gene name	Fold change	Function
Smooth muscle myosin light chain kinase	1.706	Cell Structure
Prostaglandin H synthase-2	1.433	COX
Immunoglobulin mu chain secreted form	1.77	Ig
Ig rearranged mu-chain	1.509	Ig
Gamma-globin gene, exon 2	1.507	Ig
Serum albumin	1.581	Immune/Inflammatory
Serum albumin	1.408	Immune/Inflammatory
Fetuin	1.388	Immune/Inflammatory
Complement component C3	1.339	Immune/Inflammatory
Kunitz domain protein	1.734	Kunitz
Beta-casein	1.675	Membrane
Uterine IEL TCR delta 5	1.652	Membrane
CFTR gene, intron 1	1.386	Membrane
Aldehyde dehydrogenase	1.865	Metabolic
Aldehyde dehydrogenase	1.839	Metabolic
Aldehyde dehydrogenase	1.812	Metabolic
Cytochrome P-450 17-alpha-hydroxylase	1.587	Metabolic
Aldehyde dehydrogenase	1.552	Metabolic
Aldehyde dehydrogenase	1.521	Metabolic
Aldehyde dehydrogenase	1.49	Metabolic
Cytochrome P-450 17-alpha-hydroxylase	1.453	Metabolic
Aldehyde dehydrogenase	1.434	Metabolic
MHC class I protein	1.971	MHC

MHC OVAR-DQ-ALPHA1	1.721	MHC
MHC OVAR-DQ-ALPHA-2.1	1.692	MHC
MHC class II OVAR-DQB1	1.633	MHC
MHC Ovar-DR-alpha	1.558	MHC
MHC OVAR-DQ-ALPHA-2.1	1.529	MHC
DR beta-chain antigen binding domain	1.478	MHC
MHC Ovar-DRB1	1.385	MHC
MHC class I protein	1.338	MHC
Kappa-casein	1.856	Milk
Mitochondrial	1.955	Mitochondrial
Diubiquitin	1.925	Mitochondrial
Mitochondrial	1.912	Mitochondrial
Mitochondrial	1.403	Mitochondrial
Polyubiquitin	1.343	Mitochondrial
Mitochondrial ATP synthase c subunit P2 form	1.306	Mitochondrial
Mitochondrial	1.266	Mitochondrial
Polyubiquitin	1.256	Mitochondrial
prion protein	1.372	Prion
prion protein	1.233	Prion
Cytochrome c-oxidase subunit three COIII	1.53	Respiratory
Unidentified SSH clone insert	1.923	Unidentified
Unidentified SSH clone insert	1.9	Unidentified
Unidentified SSH clone insert	1.881	Unidentified
Unidentified SSH clone insert	1.736	Unidentified
Unidentified SSH clone insert	1.644	Unidentified
Unidentified SSH clone insert	1.631	Unidentified

Unidentified SSH clone insert	1.499	Unidentified
Unidentified SSH clone insert	1.489	Unidentified
Unidentified SSH clone insert	1.473	Unidentified
Unidentified SSH clone insert	1.468	Unidentified
Unidentified SSH clone insert	1.436	Unidentified
Unidentified SSH clone insert	1.412	Unidentified
Unidentified SSH clone insert	1.395	Unidentified
Unidentified SSH clone insert	1.381	Unidentified
Unidentified SSH clone insert	1.353	Unidentified
Unidentified SSH clone insert	1.276	Unidentified
Unidentified SSH clone insert	1.234	Unidentified
Unidentified SSH clone insert	1.198	Unidentified
Unidentified SSH clone insert	1.171	Unidentified
Unidentified SSH clone insert	1.155	Unidentified
Unidentified SSH clone insert	1.135	Unidentified
Unidentified SSH clone insert	1.095	Unidentified
Mariner related transposon Hsmar1	1.615	Transposon
Mariner related transposon Hsmar1	1.419	Transposon
Unknown	1.795	Unknown
Unknown	1.755	Unknown
Unknown	1.525	Unknown

Table 6.8: Details of the genes that were classified as weakly (>0.5 fold) down regulated in the directly challenged segment at 6 hours post-LPS treatment. The derivation of the fold change and assigned functional classification was as described in Table 6.5.

Gene name	Fold change	Function
Beta actin	0.737	Cell Structure
Actin	0.735	Cell Structure
Beta-actin	0.735	Cell Structure
Activin receptor type IIB	0.559	Cell Structure
centromeric satellite I	0.574	Centromere
Placental lactogen precursor PL, exon 1	0.781	Growth Hormone
Placental lactogen precursor PL, exon 1	0.722	Growth Hormone
Immunoglobulin alpha heavy chain IgA	0.717	Ig
Immunoglobulin mu chain secreted form	0.623	Ig
Immunoglobulin gamma1 chain secreted form	0.618	Ig
Ig gamma 2 constant region heavy chain	0.558	Ig
Ferritin heavy-chain	0.786	Immune/Inflammatory
Endothelin receptor A precursor	0.722	Immune/Inflammatory
Ferritin heavy-chain	0.704	Immune/Inflammatory
Skeletal muscle-specific calpain	0.699	Immune/Inflammatory
Intercellular adhesion molecule-1 precursor ICAM-1	0.69	Immune/Inflammatory
Ferritin heavy-chain	0.672	Immune/Inflammatory
TIMP-1=metalloproteinase tissue inhibitor	0.655	Immune/Inflammatory
Interferon regulatory factor 1	0.632	Immune/Inflammatory
DAP12 protein	0.629	Immune/Inflammatory
Interleukin 6	0.622	Immune/Inflammatory
Retinol-binding protein	0.596	Immune/Inflammatory

Ferritin heavy-chain	0.589	Immune/Inflammatory
Ferritin heavy-chain	0.577	Immune/Inflammatory
Fetuin	0.562	Immune/Inflammatory
Fetuin	0.559	Immune/Inflammatory
Fetuin	0.551	Immune/Inflammatory
Histone H2A.Z	0.531	Immune/Inflammatory
CFTR gene, intron 1	0.761	Membrane
ATP 7B protein	0.703	Membrane
T-cell receptor beta-chain	0.689	Membrane
Glucose transportertype 3 GLUT-3	0.605	Membrane
Decorin	0.591	Membrane
Glucose transportertype 3 GLUT-3	0.538	Membrane
Peripheral-type benzodiazepine receptor PBR	0.597	Metabolic
O-acetyltransferase	0.573	Metabolic
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	0.57	Metabolic
MHC class I protein	0.641	MHC
Mitochondrial	0.689	Mitochondrial
Mitochondrial	0.673	Mitochondrial
diubiquitin	0.617	Mitochondrial
Mitochondrial	0.578	Mitochondrial
6-phosphogluconate dehydrogenase	0.68	Respiratory
6-phosphogluconate dehydrogenase	0.633	Respiratory
6-phosphogluconate dehydrogenase	0.517	Respiratory
Unidentified SSH clone insert	0.768	Unidentified
Unidentified SSH clone insert	0.742	Unidentified

Unidentified SSH clone insert	0.722	Unidentified
Unidentified SSH clone insert	0.719	Unidentified
Unidentified SSH clone insert	0.7	Unidentified
Unidentified SSH clone insert	0.695	Unidentified
Unidentified SSH clone insert	0.64	Unidentified
Unidentified SSH clone insert	0.638	Unidentified
Unidentified SSH clone insert	0.63	Unidentified
Unidentified SSH clone insert	0.616	Unidentified
Unidentified SSH clone insert	0.611	Unidentified
Unidentified SSH clone insert	0.605	Unidentified
Unidentified SSH clone insert	0.604	Unidentified
Unidentified SSH clone insert	0.591	Unidentified
Unidentified SSH clone insert	0.588	Unidentified
Unidentified SSH clone insert	0.577	Unidentified
Unidentified SSH clone insert	0.573	Unidentified
Unidentified SSH clone insert	0.56	Unidentified
Unidentified SSH clone insert	0.555	Unidentified
Unidentified SSH clone insert	0.542	Unidentified
Unidentified SSH clone insert	0.537	Unidentified
Unidentified SSH clone insert	0.526	Unidentified
Unidentified SSH clone insert	0.521	Unidentified
Unidentified SSH clone insert	0.51	Unidentified
Unidentified SSH clone insert	0.501	Unidentified
Oligodendrocyte GTP-binding protein	0.524	Transcription factor
WBC STS genomic, sequence tagged site	0.613	Unknown

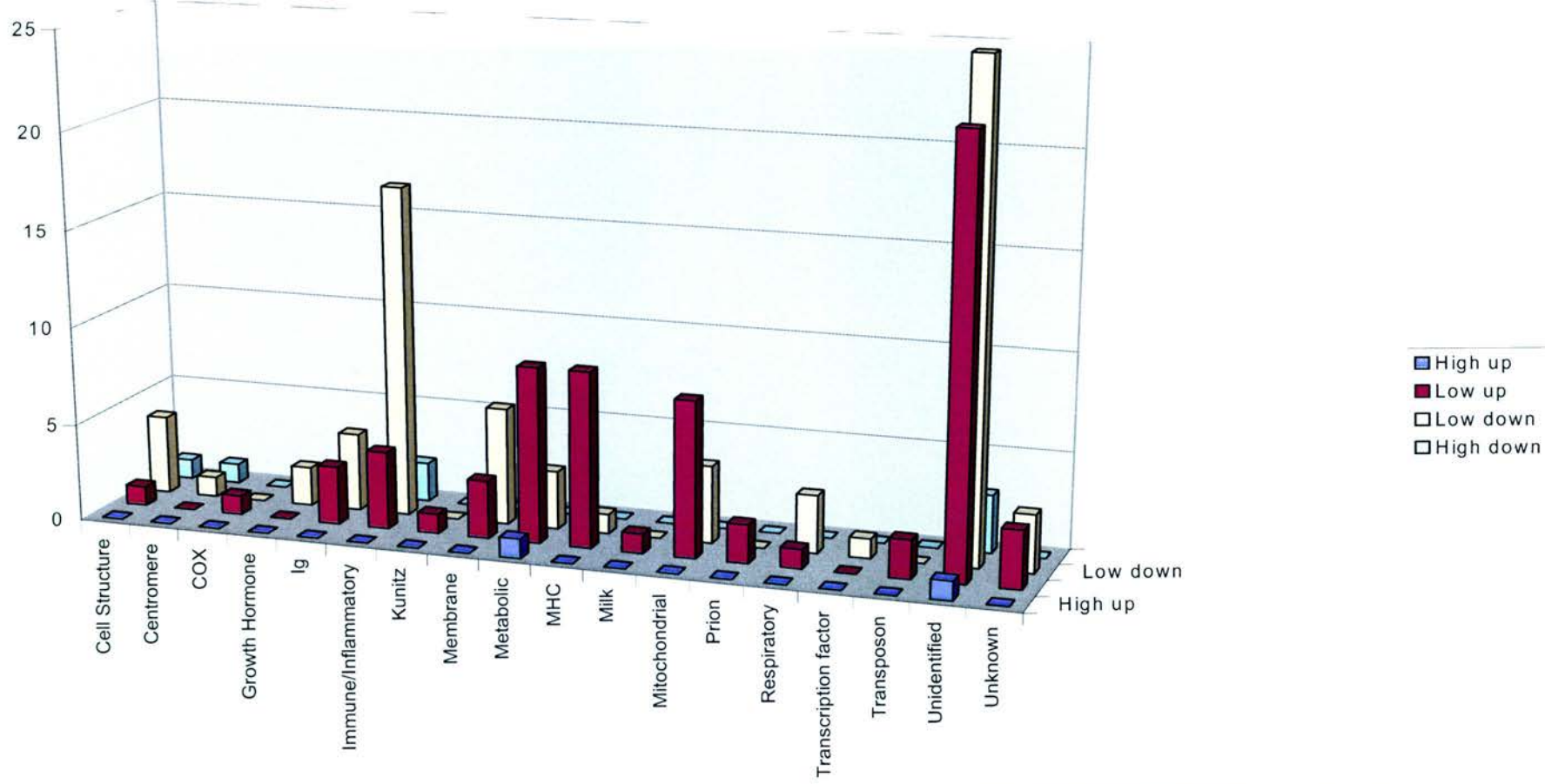


Figure 6.7: 3D bar chart displaying the number of elements showing significant regulation in the directly challenged lung segment 6 hours after LPS challenge. The elements were grouped according to function (x axis) and also according to expression change (z axis). The number of elements represented in each of the resultant groups of was then plotted on the y axis. The height of each column therefore represents the number of elements in that function/expression group. The highest peaks are those representing the unidentified function elements, this is due to the reduced numbers of sequenced SSH elements in this segment. Of the identified elements immune/inflammatory (low down), metabolic, mitochondrial and MHC (low up) and cell structure and membrane (low down) are the most prominent peaks. Growth hormones were all down regulated at low levels.

Direct vs. Remote comparison

The different expression levels of each element present in the normalised data between the direct and remote segments were plotted against each other and are shown as a scatter graph in figure 6.8. The majority of elements are found around the 1-fold mark, indicating similar expression between the direct and remote segments. There were more genes altered in the remote segment than in the direct segment.

RNA quality and amplification

Table 6.9 details the quantity of amplified RNA obtained from each sample taken from the brushed epithelial cells. Figure 6.9 displays representative 'false gel' images that were used to distinguish poor quality RNA for exclusion. Unfortunately a mechanical fault destroyed a proportion of the RNA samples prior to amplification, and consequently fewer hybridisations were achieved than were planned. There were 8 sheep samples used from the remote segment, and 5 from the directly challenged segment. Each sample was hybridised in duplicate with the dyes swapped to avoid any errors based on differential dye fluorescence. Full analysis of the amplified RNA is presented in Appendix III.

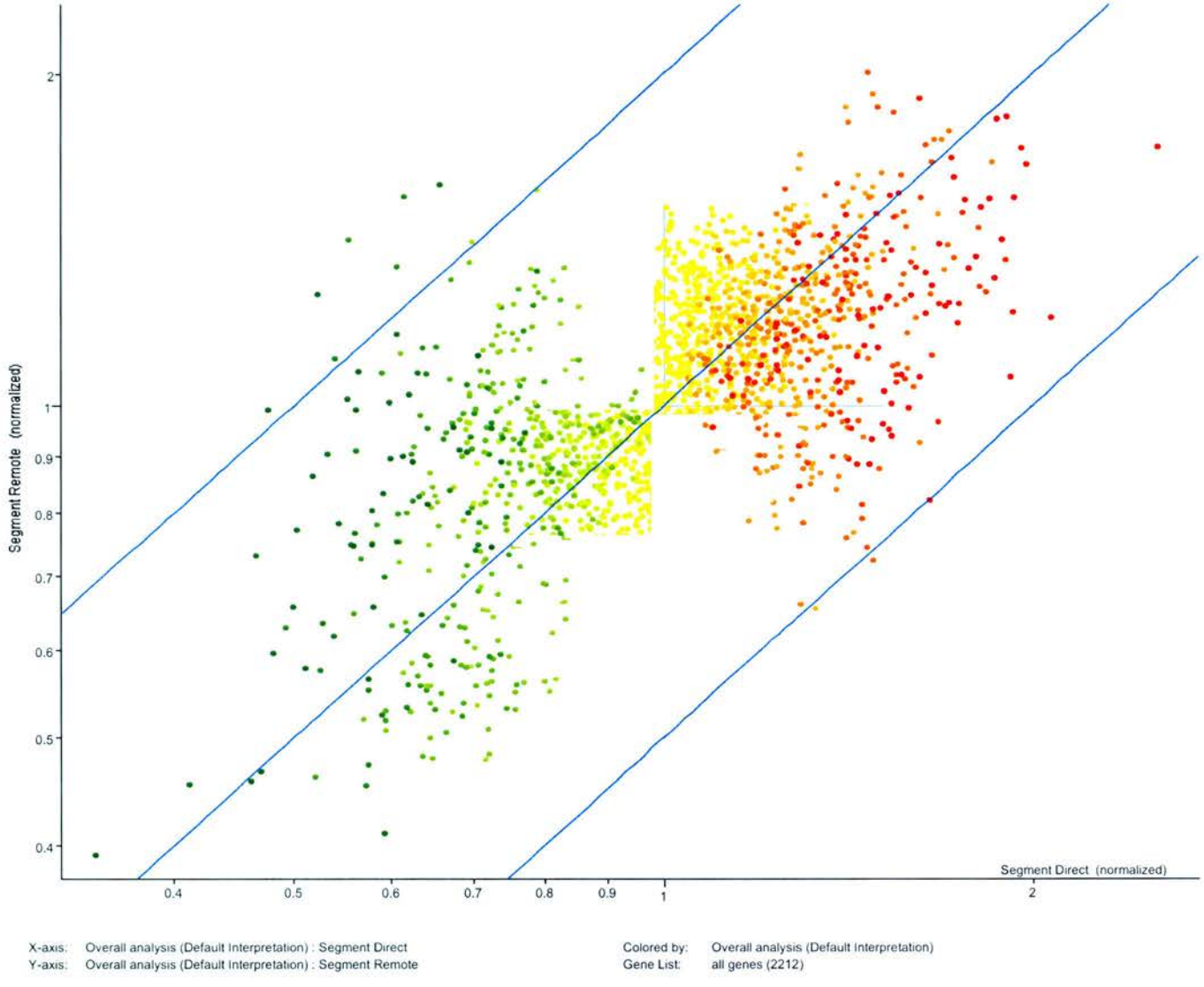


Figure 6.8: Scatter plot of each microarray element's normalised change in fluorescence intensity in the contra lateral lung (x axis) vs. directly challenged lung segment 6 hours post-LPS challenge (y axis). Blue lines (from left to right) represent approximately 2, 1, and 0.5-fold difference respectively. Each point on the graph represents an element on the array, and is coloured according to significance, i.e. the more intensely red the point, the higher the significance in the direct segment is, whilst the more intense the green colour, the higher the significance in the remote segment is. Elements that have a weaker colour show low levels of statistical significance. The majority of elements are not significantly different in fold change between direct and remote segments. The majority of elements cluster in the centre as expected.

*Table 6.9: Quantities of amplified RNA (μg) produced from the original total RNA samples extracted from brushed epithelial cells derived from each sheep lung sample. Direct samples were taken from the LPS-challenged segment, remote from the contra lateral lung segment at baseline (0 hours) or post-LPS challenge (6 hours). The quantification of the RNA was performed using the Agilent Bioanalyzer platform which calculates RNA concentration using fluorescence-based detection technology. The original RNA was purified and divided into two. The first half of each sample was used for real-time quantitative RT-PCR analysis of cytokines. The second half was used for amplification and labelling prior to microarray hybridisation. Samples labelled * were in a pair of samples not sufficient for microarray hybridisations, those spaces labelled ** were samples which were excluded through technical error or insufficient quality of total RNA.*

Sheep	Direct (0h)	Direct (6h)	Remote (0h)	Remote (6h)
O687P	50.09	28.42	**	**
O693P	**	**	22.15	16.42
O861P	11.13*	1.41*	15	15
O681P	31.79	11.02	16.2	15.8
O631P	26.86	4.14	18.6	18.6
O757P	15	15	14.2	24.2
S46	52.16	27.18	39.6	32.6
X26	**	**	14.8	9
S41	**	**	30	30

Figure 6.9.A: A 'false gel image' representing total RNA samples purified from brushed epithelial cells from lung segments. The image was derived from the fluorescence trace data acquired by the Agilent Bioanalyzer chip platform. The analysis software extrapolated bands from corresponding peaks in the fluorescence trace data. RNA quality was assessed visually using both these gel impressions and the trace data in order to exclude poor quality RNA samples. The presence of clear ribosomal peaks indicates intact RNA is present.

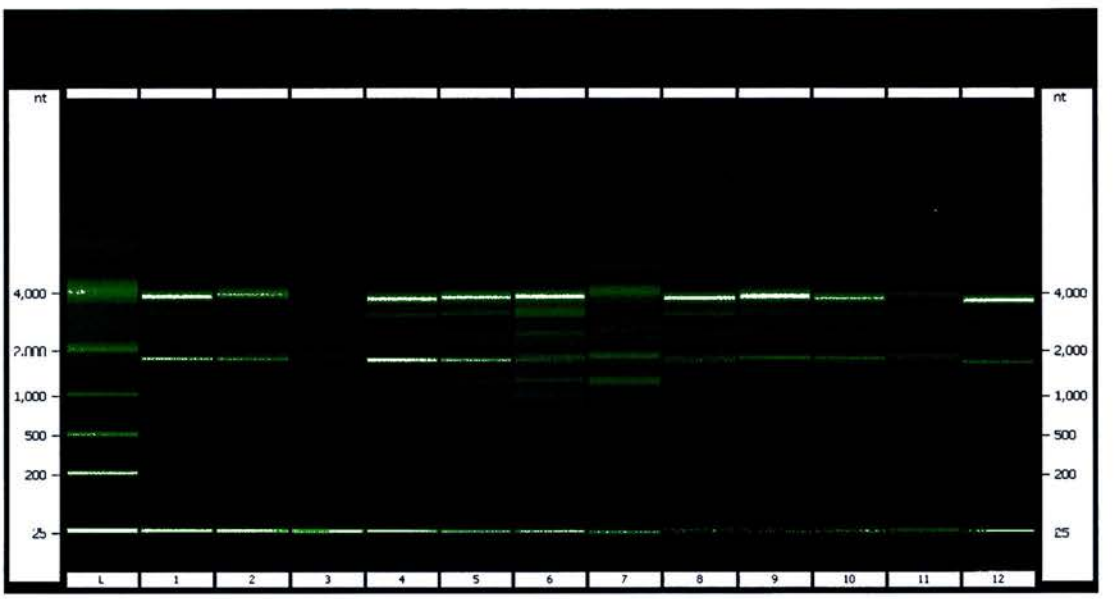
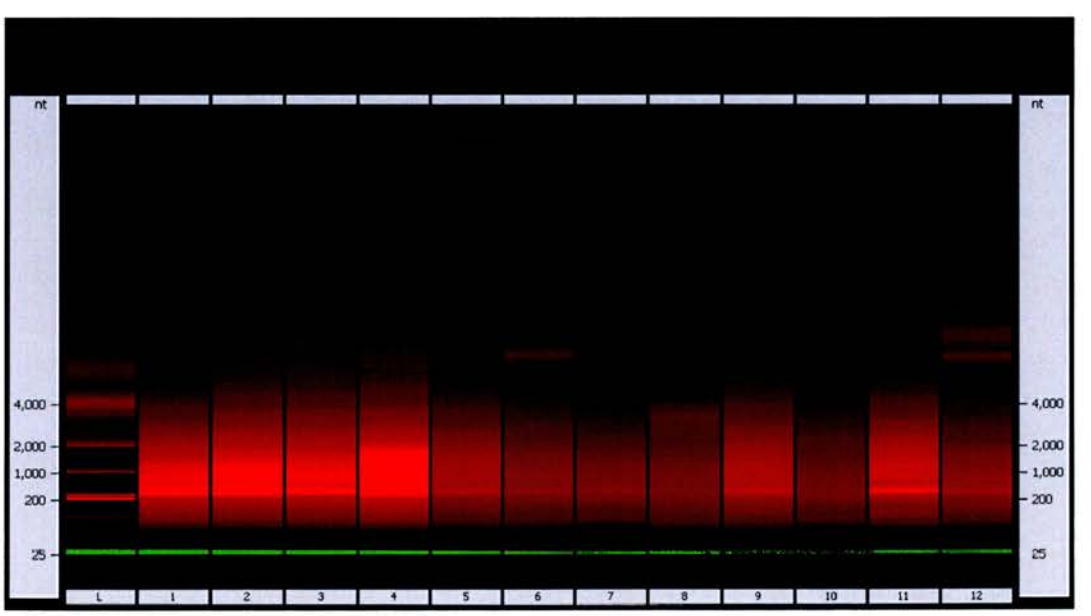


Figure 6.9.B: A 'false gel image' representing amplified aRNA samples from original RNA purified from brushed epithelial cells from lung segments. The image was derived from the fluorescence trace data acquired by the Agilent Bioanalyzer chip platform. The analysis software extrapolated bands from corresponding peaks in the fluorescence trace data. RNA quality was assessed visually using both these gel impressions and the trace data in order to exclude poor quality RNA samples. The lack of ribosomal peaks indicate that there was no ribosomal RNA contamination of the samples after amplification.



Discussion

Remote segment gene expression

There appear to be a number of gene networks active in the contra lateral segment following local LPS challenge. These networks are associated with a number of functions, some apparently unrelated to inflammation or immune responses.

There were changes in the regulation of a considerable number of ribosomal proteins, which divide relatively evenly between low levels of up and down regulation. Elements corresponding to the small ribosomal subunit proteins S23a and S25 exhibit increased expression in the remote segment following LPS, whilst those corresponding to the large ribosomal subunit proteins L30, L32 and L35 and the small ribosomal subunit proteins S16, S12, S23, S25, S27a and S7 exhibit decreased expression following LPS challenge.

Ribosomal proteins are major constituents of ribosomes that catalyse protein synthesis in the cytoplasm (Mager 1988). The catalytic functions specific to individual ribosomal proteins are largely unknown (Wool 1996; Wool, Chan, & Gluck 1995). Under normal conditions, ribosomal proteins are synthesized with rRNA to produce RNA and protein. Under altered conditions, including events surrounding cellular growth and proliferation, the expression levels of ribosomal proteins are altered. For instance, both the large and small ribosomal subunit proteins 7, 23a and 27a exhibited decreased expression in a study of neuronal differentiation of human embryonic carcinoma cells (Chester et al. 1989), this correlated well for levels of the small ribosomal subunit proteins in a further study (Bevort & Leffers 2000). These alterations appear to be restricted to specific ribosomal proteins, because some ribosomal protein levels are altered, whereas others remain constant (Frigerio et al.

1995). The contradictory evidence for ribosomal subunit protein S25 demonstrating both increased and decreased regulation may be due to very similar transcripts being differentially regulated and cross-hybridising to both elements.

Independent alterations in ribosomal protein synthesis suggest that individual ribosomal proteins have functions beyond the simple structural makeup of the ribosome or protein synthesis. For example, P0 and S3 possess endonuclease activity, suggesting that they may have DNA repair functions (Grabowski et al. 1991; Kim et al. 1995), and subunit 7 can function as a co activator of nuclear receptors (Berghofer-Hochheimer et al. 1998) and has been implicated in apoptosis (Goldstone & Lavin 1993; Naora et al. 1998; Neumann & Krawinkel 1997). Altered ratios of ribosomal subunits are also associated with stage-specific tissue development (Mazuruk et al. 1996).

The present data provide evidence that the levels of specific ribosomal transcripts are altered in the contra-lateral lung following local LPS challenge. The results suggest that regulation of protein synthesis and/or other functions mediated by ribosomes are associated with the remote response in the lung.

This could potentially indicate that the cells were in the process of closing down non-essential functions – possibly in preparation for a rapid response to any incipient exposure to a stimulus, whilst boosting functions that may be needed to respond to a challenge. Another possibility raised by the regulation of ribosomal proteins is that the cells were preparing for apoptosis (Goldstone & Lavin 1993; Naora et al. 1998; Neumann & Krawinkel 1997).

Cell structure genes, including actin binding genes, actin itself and actin regulator genes such as filensin and thymosin beta, appeared to be down regulated in contra-lateral sites. Actin-based structures participate in the regulation of cell motility,

growth and differentiation, not only by altering cell morphology, but also by acting as active components of the signalling complexes transmitting signals to the nucleus and thereby regulating gene activation (Vicente-Manzanares et al. 2002; Bamburg & Wiggan 2002).

The role of actin and associated cytoskeletal genes and gene products has not been extensively investigated in the context of pulmonary inflammation. Studies specifically investigating the expression of this category of molecule have demonstrated involvement in the migration of neutrophils in a rabbit model of bacterial pneumonia (Mueller et al. 1994). In this model, the inhibition of actin de-polymerisation resulted in reduced trafficking of neutrophils from the circulation into the lung tissue following bacterial challenge, and that this was due to actin rearrangement in the lung cells and not the neutrophils themselves.

Relatedly McClenahan et al investigated the expression of filamentous actin in bovine neutrophils in response to a variety of stimuli including LPS (McClenahan et al. 2000). This study concluded that filamentous actin played a critical role in altering the deformability of neutrophils *in vitro*, though there was no significant alteration in actin expression as a result of bacterial products. Lim et al also demonstrated that actin plays a role in the reaction to inflammatory stimulus in pulmonary microvasculature (Lim et al. 2001). This study concluded that inflammation results in considerable actin rearrangement associated with alterations in expression and spatial localisation of other cytoskeletal factors.

These studies support the assertion that observed down-regulation may relate to a change in cellular morphology within the airway epithelium associated with neutrophil trafficking and sequestration. Whilst the mechanism of action and biologic consequences of these alterations remain unclear, the central role of neutrophil

movement in the inflamed lung in terms of bacterial clearance and tissue damage together with the data from this study warrant further investigation into the role of the cytoskeleton in pulmonary inflammation.

A large number of **mitochondrial genes** were significantly regulated in the remote segment in response to local lung administration of LPS, though their pattern of regulation was unclear as similar numbers of elements were increased and decreased. **ATPase 6** in particular represents a large proportion of the elements altered and, though it is unclear whether it is up or down regulated, the regulation is at very low levels. This observation is of particular note as this gene has been implicated in the inflammatory response to *Pneumocystis carinii* infection in the rat lung (Asnicar et al. 2001). This study demonstrated up regulation of a fragment of the ATPase 6 gene in whole lung compared with control lung by differential display, and also localised the expression to Clara cells and type II pneumocytes.

Diubiquitin was also regulated significantly, this protein consists of two ubiquitin-like molecules arranged in a head to tail formation and its expression has been shown to be induced synergistically by a combination of interferon γ and TNFα (Raasi et al. 1999), both of which are generally accepted mediators of the acute inflammatory response in the lung. Although the functional role of this molecule has yet to be elucidated, structurally similar molecules have been implicated in the inhibition of the ubiquitin/proteosome pathway (Swinney et al. 2002). This latter pathway is involved in initiating NFκB transcription which in turn leads to cytokine production in response to LPS signalling. These results may suggest that the epithelial cells were altering the mitochondrial output as a part of the inflammatory cascade, though the means and ends of these expression changes are unknown.

Genes expressed in membranes were also significantly affected. These included MHC molecules, cluster designation (CD) cell markers, transport molecules and extra-cellular matrix molecules. Genes involved with T-cell recognition and MHC presentation, namely **MHC class I proteins** and **T-cell receptor beta chain**, were significantly up-regulated, whilst decorin, involved with cell signalling via the extra-cellular matrix (Tran, Griffith, & Wells 2004), solute carrier (a transport molecule), and the surfactant proteins A and C were all down regulated.

The up regulation of the T-cell associated genes could potentially imply that the organ was initiating the adaptive immune response cascade, and it would be reasonable to hypothesise that this is in response to signals received from the directly challenged segment of the lung. This would also concur with the results seen in chapter 4 where IL 10 was up regulated in the remote segment. IL 10 has been shown to have a central role in the switch from innate to adaptive immunity (Mocellin et al. 2004; Yates et al. 2000; Bonig et al. 1998).

The down regulation of the **surfactant protein** (SP) genes is of note as these have been implicated in the pathogenesis of ARDS (Balamugesh et al. 2003). SP-A is a member of the collectin family, which are pattern recognition models recognised as mediators of innate and adaptive immune functions (review: (Wright et al. 2001)). In a study on SP-A expression in a clinical cohort of ARDS patients a lower level of the protein was significantly associated with an infectious aetiology as opposed to a non-infectious one (Balamugesh et al. 2003). A further study using repeat endotoxin challenge associated the increased expression of SP-A in whole lung with direct endotoxin challenge (George et al. 2003).

SP-C meanwhile has been demonstrated as a protective factor in oxygen lung injury (Ikegami et al. 2002), and its down regulation may be an anti-inflammatory reaction

that renders the lung tissue more susceptible to inflammatory mediated tissue injury as is implied by the results of a study by Mikawa et al demonstrating a protective role for this protein in acid-induced lung injury in an intratracheal rabbit model (Mikawa et al. 2004).

A number of genes associated with immune and inflammatory activity were altered. **Interferon γ associated genes** (interferon gamma-inducible-protein 10, Kunitz domain protein and prostaglandin endoperoxidase) were significantly up regulated as a group within these elements, which might suggest a role for IFN signalling in the promotion of the remote response – this is consistent with the remote IFN signalling reported by Halloran (Halloran et al. 1992) in a study which found increased expression of numerous genes in remote organs and distant parts of inflamed organs during rejection of an ascites tumour allograft and skin sensitisation by oxalozone. The blockade of IFNγ in the above study reduced remote regulation following inflammatory stimulus suggesting that the IFNγ pathway may constitute a potential regulatory mechanism at a distance from localised stimuli.

Other Immune and inflammation associated genes altered in the remote segment include **complement protein C3.** This is the initiator molecule for the alternative complement pathway and is heavily implicated in the immune reaction to gram negative bacteria (Mueller-Ortiz, Drouin, & Wetsel 2004; de Astorza et al. 2004; Shankar-Sinha et al. 2004; Pishko et al. 2004; Younger et al. 2003). A recent study by Meuller-Ortiz et al found that C3 knockout mice were significantly more at risk of mortality due to *Pseudomonas pneumonia* than wild-type mice (Mueller-Ortiz, Drouin, & Wetsel 2004), a result confirmed in principle by Younger (Younger et al. 2003). *In vitro* studies have demonstrated an opsination-based mechanism of innate defence against bacterial infection by alveolar epithelial cells in an A549-based cell

line study (de Astorza et al. 2004). The increase in expression of this molecule hints at a localised protective response contra-lateral from the focus of inflammation, an event that has not been reported previously.

Trypsin inhibitor expression was also up regulated in the remote segment. Schmek et al recently demonstrated that inhibition of trypsin-like proteases reduced apoptosis of human alveolar and bronchial epithelial cells *in vitro* in response to pneumococcal infection (Schmeck et al. 2004). In addition, Thomas et al have reported that trypsin impairs adherence of *Mycoplasma bovis* to bovine epithelial cells *in vitro* (Thomas et al. 2003) and Tamura et al have demonstrated that trypsin inhibits the adherence of group B *Streptococci* to the epithelial A549 cell line (Tamura et al. 1994). These studies would indicate that increased expression of a trypsin inhibitory molecule might result in an increased susceptibility to bacterial colonisation in the lung.

Osteopontin - a multifunctional matricellular protein abundantly expressed during inflammation and repair – was significantly down regulated in the remote segment following LPS treatment. A recent microarray study on osteopontin null mice indicated that osteopontin is a critical factor in the development of fibrosis in the murine lung, and that the epithelium is the major cellular source of the molecule during murine pneumonitis and bleomycin-induced fibrosis (Berman et al. 2004). This study is consistent with others reporting a central role for osteopontin in pulmonary fibrosis, as well as in other immune and inflammatory responses in the lung and other organs (O'Regan 2003; Ortiz et al. 2003; Takahashi et al. 2001).

Prostaglandin D2 synthase (PG (D2)S) is a key enzyme in the generation of the prostaglandin J and D series of molecules which are heavily implicated in immune and inflammatory responses (Ando et al. 2003). PG (D2)S product prostaglandin J2 has been recently studied by Inoue et al in a mouse model of LPS intratracheal

instillation (Inoue et al. 2003). This study presented convincing evidence that, in the murine LPS instillation model, PG J2 had significant pro-inflammatory effects, increasing the expression of the classical inflammatory mediators interleukin 1β, macrophage inflammatory protein-1α, and macrophage chemoattractant protein-1 as well as significantly increasing the degree of neutrophilic inflammation and oedema in the lung in response to LPS. The reduced expression of PG (D2)S in the remote segment would imply an anti-inflammatory response to LPS in the contra lateral lung.

Alpha-s1-casein expression was also significantly reduced in the remote segment, this is intriguing as there is no published evidence of this gene's regulation or even expression in the lung, and its main function is generally accepted to be in milk production (Karatzas & Turner 1997; Klei et al. 1997). The expression of alpha-s1-casein has thus far been reported in experimentally induced bovine mastitis (Le Roux, Laurent, & Moussaoui 2003), though it also serves as an inflammagen in its own right (Mullner, Lazar, & Hrabak 2002; Vermehren et al. 2001). The latter use of casein as an inflammatory inducer may indicate that, if constitutively expressed, it acts as a pro-inflammatory mediator and its suppression seen as an anti-inflammatory effect, however this is purely speculative and requires further investigation.

Enzymes associated with cellular respiration were significantly regulated also. Down regulated elements in the remote segment include cytochrome c oxidase II, and Kreb's cycle associated enzymes, whilst the cytochrome c oxidase I and III subunits were up regulated. These alterations may indicate an increase in nitric oxide catabolism (Brunori et al. 1999) or an adaptation to a shift in specific energy needs (Cheng et al. 2003; Villani et al. 1998; Chandel et al. 1995). The mitochondrial gene products represented by the sequences affected in this study may also play a role in the generation of reactive oxygen species (ROS). Indeed Kuwano et al demonstrated

significant alteration of the overall mitochondrial mass in human epithelial cells from clinical patients as a result of idiopathic interstitial pneumonia (Kuwano et al. 2003) and a corresponding increase in the level of ROS production.

Transcription factors were also affected, with 2 signal transducer and activator of transcription (STAT) factor homologs (labelled as chromosome 18 sequences) up regulated and Ets-2 factor down regulated. DNA-binding STAT transcription factors have been reported to play a role in allergic lung disease (Kotsimbos et al. 1997) where they are critical mediators of the IL 5 directed eosinophil responses (Mishra & Rothenberg 2003; Taha et al. 2003; Yang et al. 2001). Ets-2 is poorly characterised in the lung, though a closely related protein, Elk-3 has been implicated as a transcriptional repressor of nitric oxide synthesis in a mouse model of endotoxaemia (Chen et al. 2003). Ets-2 also has a suggested role in the regulation of IL-12 in an inflammatory response in macrophages and in arthritic synovial inflammation (Kawabata et al. 2004) though its mode of action and effects are unclear from these studies.

The **lipid and steroid metabolism mediators** muscle-type carnitine palmitoyltransferase I, lipoprotein lipase and cytochrome p450 17c were up regulated in the remote segment 6 hours after contra-lateral local LPS challenge. Concurrently, metabolic mediators ferritin (an iron chelator) and lectin precursor were down regulated in the same segment.

Carnitine palmitoyltransferase (CPT) and lipoprotein lipase are not well represented in the literature relating to lung biology, however both were shown by Feingold et al to be down regulated in the acute phase response to LPS in a mouse heart disease model (Feingold et al. 2004). This concurs with observations in a mouse sepsis model by Eaton et al (Eaton et al. 2003) and a rat inflammation model using

caecal ligation puncture by Bazel et al. (Bazel et al. 1999). This latter study was of interest in terms of bacterial inflammation as it demonstrated that sterile inflammation failed to inhibit CPT expression whereas caecal ligation puncture significantly inhibited it, thus implying a role for infecting bacterial compounds in the inhibition of CPT in this model. The increases expression of CPT in this study would seem to correlate well with the other observations that the remote response is contrary to that seen in direct inflammation in various models.

Lipoprotein lipase, likewise, has been implicated in the response to sepsis in host species. A review by Khovidhunkit et al of the effects of infection and inflammation report a significant inhibition of lipoprotein lipase in sepsis (Khovidhunkit et al. 2004) and attribute to it a protective role against septic sequelae, this study is supported by findings in a study by Harris et al who also show inhibition of lipoprotein lipase in LPS-induced sepsis leading to 'lipemia of sepsis' (Harris, Gosnell, & Kumwenda 2000). The production of lipid and steroid mediators could be perceived as an anti-inflammatory or protective response to counter inflammation, though this conjecture requires considerable investigation to determine what, is any, biological impact their regulation might have in the progression of lung disease.

Cytochrome P450 (CYP450) has been studied in some depth due to its effects on drug metabolism. Whilst its biological effects in the lung remain to be studied in detail, it has been established by numerous studies that CYP450 mediated drug metabolism is inhibited by direct inflammatory stimulus (Carcillo et al. 2003; Saitoh, Kokue, & Shimoda 1999; Monshouwer et al. 1996). However the direct effect of LPS on CYP450 expression is not clear as tissue expression of the molecule and its products have been both increased (Tindberg et al. 1996) and decreased (Shimamoto et al. 1998) in rats following inflammatory stimuli. CYP450 has not been reported to

play a direct role in the inflammatory cascade, but certainly appears to be regulated by its products (Bleau et al. 2003).

Ferritin, whose expression was decreased in the remote segment of the sheep lung in this study, is an iron chelator whose primary function in infection is to withhold iron from colonising bacteria (reviewed by Jurado (Jurado 1997)). Though its effect in lungs are poorly studied, it has been shown to play a role in the increased bacterial load in lungs of patients with cystic fibrosis (Reid et al. 2002). Inflammatory infection appears to be associated with increased ferritin levels in human pus and serum samples (Bryant, Crouse, & Deagen 2004; Tomkins 2003), but its regulation in tissue following direct inflammation is unclear.

The reduction in expression of **lectin precursor** also adds to this evidence. Lectins are critical in lung inflammation and infection as binding and recognition molecules (Sato et al. 2002; Pavia-Ruz et al. 1994; Mariassy et al. 1989), and their expression is closely linked with that of the surfactant proteins, which are a part of the C-lectin family (Kuroki & Sano 1999), in response to bacterial infection (Leth-Larsen et al. 2003). The similar expression profile of lectin precursor to the surfactant proteins in the remote segment supports this functional link.

Prostaglandin genes appeared to be consistently regulated. The prostaglandin endoperoxidase and prostaglandin endoperoxidase associated receptor (Em4b) were significantly up regulated and prostaglandin D2 synthase was significantly down regulated. This concurs with observations that PGE2 and PGD2S have contrasting effects in lungs (Zeldin et al. 2001). These genes are targets for non-steroidal ant-inflammatory drugs (Colville-Nash & Gilroy 2001) and are therefore considered to be pro-inflammatory in nature, however it has also been suggested that they can exert anti-inflammatory effects *in vivo* (Gilroy et al. 1999).

Ovine **growth hormone** was significantly up regulated. Growth hormone has been associated with inflammatory marker reduction in acute inflammation (Wu, Herndon, & Wolf 2003; Basoglu et al. 2002; Bergad et al. 2000; Derfalvi et al. 2000) but its mode of action is unclear (Hansen 2003).

A number of genes were altered which were either of unknown function, or could not be identified as homologs of known genes in comparative species, and these constitute a considerable proportion of the data (29%).

Direct segment gene expression

In common with the remote segments, MHC and T cell receptor genes represented a significant group of up regulated elements in the directly instilled segments.

Mitochondrial gene products (diubiquitin, polyubiquitin, ATP synthase) showed a greater trend to be up regulated in the direct segment than in the remote, though this trend was marginal. Similarly the metabolic elements (e.g. 6-phosphogluconate dehydrogenase, cytochrome P450) showed slightly more up regulation in the direct than the remote segment. The pattern of metabolic functions altered in the direct was similar to the remote with genes concerned with steroid and lipid metabolism representing the majority of those up regulated. The steroid metabolic enzyme cytochrome P450 17c, notably, demonstrated 1.7 fold up regulation in the direct segment. Cell structure elements were down regulated in the direct segment - as was found in the remote.

A notable difference between the direct and remote segments was observed in relation to the expression of immune and inflammatory elements. In the direct segment there were a considerable number of down regulated inflammatory regulators such as fetuin (Demetriou et al. 1996), peripheral-type benzodiazepine receptor (Bribes, Bourrie, & Casellas 2003) and histone H2A.Z – all of which have been shown to be cytokine

inhibitors, and whose down regulation could potentially enhance the inflammatory response of the tissue.

Up regulated immune genes include prostaglandin H2 synthase (Zeldin et al. 2001) and complement component C3 (Younger et al. 2003; Walters et al. 2002; Drouin et al. 2001; Ault & Colten 1994), both of which are recognised inflammatory products. Interestingly elements corresponding to inflammatory markers were also seen to be down regulated, this included interferon regulatory factor 1, metalloproteinase tissue inhibitor 1, interleukin 6 and intercellular adhesion molecule 1. Although it is conceivable that these genes reached peak levels prior to 6 hours and were then down regulated, it should be noted that the result for IL 6 is contradictory to that seen in the real-time PCR experiment from chapter 4.

This discrepancy is initially concerning given the direct contradiction between the two methodologies, however, a number of possible confounding factors must be considered in the context of the microarray data. The use of a bovine analogue to the ovine IL 6 gene sequence renders this result somewhat susceptible to cross-hybridisation interference which might be a product of similar sequences being expressed in the ovine genome.

Other possible confounding factors include non-specific binding to this spot as a result of high-affinity binding motifs in the IL 6 sequence, or a highly expressed alternative splice form being expressed in the baseline samples which would be specifically excluded using the PCR methodology but might be picked up with the less discriminatory microarray platform. Regardless of these potential errors, the validation of the microarray data should engage the question of the IL 6 data directly, as well as confirming other sequences within the data set presented.

The growth hormone gene was down regulated in the direct segment, whilst in the remote segment these hormones showed an increase in expression. This result is consistent with previous studies indicating an anti-inflammatory role for growth hormone in lung inflammation (Wu, Herndon, & Wolf 2003; Derfalvi et al. 2000).

The results from the direct segment were analysed without using a false discovery rate correction method. False discovery rate correction methods are utilised to account for the relatively high expected false positive count inherent in utilising standard statistical confidence limits in large data sets. Their use typically adds to the statistical robustness of microarray data (Reiner, Yekutieli, & Benjamini 2003; Tsai, Hsueh, & Chen 2003;Y ang et al. 2003).

In the current context, use of a false discovery rate correction method effectively eliminates all significant data from the analysis of the direct segment. Whilst excluding such correction from the analysis introduces the possibility that a number of false positive results are being identified in the direct response to LPS it does avoid the tendency to generate false negatives that use of these techniques introduces (Yang et al. 2003). The choice to continue with data analysis in the absence of the false discovery rate correction was made with the assumption that any changes identified as significant would at least reflect an underlying trend in the data and could therefore be compared in a qualitative sense with the response in the contra lateral lung.

Direct vs. Remote

The overall expression profiles in the direct and remote segment s were very similar, and very few elements showed high levels of change in either a positive or negative direction. Whilst it is certainly true that microarray data analysis consistently introduces a bias towards underestimation of gene change as compared with methods such as PCR and that the robustness of the data analysis required as part of the

standard approach to microarray experiments reduce the number of sequences detected as significant, it is equally true that the data acquired from this investigation show unusually low levels of regulation of the array elements.

A further limitation in these data is the lack of apparent significant regulation of the inflammatory markers normally associated with acute inflammation in the direct segment, and the absence of the cytokine changes seen in the real-time PCR analysis of cytokine changes in either segment. The similarity in expression profiles between direct and remote segments despite the very different phenotypic changes may be explained in terms of fundamental biology.

The genetic changes involved in the two segments appear to mostly be concerned with baseline, non immune-specific alterations such as refocusing of the protein manufacturing systems and redirection of the cell's energetic make-up. These alterations are likely to underpin a very wide variety, if not most, cellular reactions in any system in the organism. Whether the cell requires an anti-inflammatory response or a pro-inflammatory one, the basis of the response will be a change in the cellular machinery that governs the manufacture of the effector molecules, with the result that the bulk of the molecular change is homogenous throughout the organism and across stimuli.

The minority of molecules that are altered therefore represent a very small, but highly potent, subset of those probed by this method. This is in contrast to single-gene, specific analyses such as the RT-PCR used in the previous chapter which look at members of that small group of effector molecules and thus show much more dramatic changes in expression. Previous array studies support this proposition, with the majority detailing changes in similar groups of genes as in this one (i.e. ribosomal proteins, steroid metabolic catalysts, mitochondrial products cell cycle determinants

and cell structure products) but in such disparate model systems as cardiac myocyte hypertrophy, colorectal cancer (Otsuka et al. 2001) and cardiac angiotensin treatment (Larkin et al. 2004).

Conclusions

Further validation of specific sequences of interest identified by this array experiment will allow more certain statements to be made about the nature of the remote response in the sheep lung, and hypothesis-driven investigation of the networks highlighted in this study may prove important in elucidating novel mechanisms of intervention in inflammatory lung disease, and particularly the evolution from localised inflammation to the fulminant organ-wide inflammation characteristic of ARDS. Further validation of the array data should comprise sequence specific techniques such as RT-PCR or related blotting methods, as well as location specific techniques such as *in situ* hybridisation methods. It is unfortunate that time constraints prevented these further analyses from being carried out within the context of this investigation, however the conclusions reached as a result of the study as concerns the hypothesis stated remain valid without need for further experimental work.

Overall the microarray results show that there is a significant, complex and wide ranging remote response and that this response is amenable to characterisation using cDNA microarrays. The data hint at a network of highly complex genetic and molecular interactions whose exact nature remains to be clarified. Though microarray data are capable of standing alone as investigative results without external verification being necessary, the possibility of cross-hybridisation with non-specific sequences interfering with the signal obtained from each element cannot be discounted.

Further verification of individual data of interest should comprise specific transcript analysis such as RT-PCR or Northern blotting. These methods are preferable due to

their specificity for the elements investigated. Microarray probes based on cDNA sequences suffer from the lack of specificity inherent in using such long stretches of sequence, thus data obtained can indicate overall changes but individual gene reactions of interest should be investigated separately using tools that exclude interference or cross-hybridisation. Such verification was beyond the scope of the present study.

The statistically significant results obtained are particularly notable due to the *in vivo* approach used, and the associated wide natural variability in gene expression inherent in using out bred sheep reared in a commercial environment.

Chapter 7 General discussion

The results obtained from the analysis of phenotypic changes in the sheep lung following direct instillation of *E. coli* LPS to a discrete segment support the assertion that the 6 hour post-LPS ovine model is an appropriate one to mimic gram negative bacterial sepsis in comparative species, including human (Monton & Torres 1998). Indeed, these results indicate that the changes in lung phenotype, particularly the cellular and histopathological changes were consistent with those previously reported due to clinical gram-negative infection (Dehoux et al. 1994; Shimabukuro, Sawa, & Gropper 2003) and currently accepted animal models (Kumar 1995).

The 6 hour LPS instillation model in sheep is thus far unreported in the available literature, and this study establishes for the first time that it is appropriate for the study of acute lung injury, a pre-requisite for studying the pathogenesis of sepsis-induced pulmonary ARDS. No phenotypic changes were found in the segment contra-lateral from the challenged segment, indicating that this is a suitable model for local infection and relates well to similar segmental challenge models in terms of phenotypic changes (Dehoux et al. 1994).

Investigation of the cytokine expression levels in the lung segment directly stimulated with LPS showed changes in cytokine levels consistent with previously published LPS inflammatory model studies and gram negative infection studies (Dehoux et al. 1994; Monton et al. 1999; Shanley, Warner, & Ward 1995). Classic inflammatory markers associated with innate immune derived responses were shown to be up regulated significantly in the instilled segment. These cytokine markers (IL 1β, IL 6 and TNFα) are widely accepted as being associated with neutrophil infiltrates in the lung (Xing et al. 1999; Toews 2001; Moore & Standiford 1998) and have been shown

to peak at between 6 and 24 hours in mouse lungs following LPS instillation (Ulich et al. 1991).

The failure to demonstrate significant changes in IL 8 expression was unexpected but may reflect a lack of expression in ovine epithelial cells in vivo. Whilst the cytokines investigated are considered to be the 'classical' molecular markers of the innate inflammatory response (Moore & Standiford 1998), there are other molecules which could have been considered as candidates for inclusion in this study. Notable amongst these are interferon gamma and IL-12, both of which play pivotal roles in the innate immune cascade in response to LPS (Heremans et al. 2000; Zisman et al. 1997; Gladue et al. 1994). Practical issues relating to cost and availability of validated primer sequences governed such decisions to reject both IL-12 and interferon gamma.

In the remote segment the inflammatory markers showed no significant changes, as would be expected and corresponding to the lack of neutrophilic infiltrate seen in the segment. The cytokine IL 10 however was significantly up regulated approximately seven fold. This change in the level of IL 10 conclusively establishes the ability of the lung to react on a whole organ basis to localised inflammatory stimulus independent of any phenotypic change. IL 10 is associated with anti-inflammatory activity and the switch from innate to adaptive immunity (Quinn et al. 2000; Raychaudhuri et al. 2000; Shanley, Vasi, & Denenberg 2000; Thomassen, Divis, & Fisher 1996; Bonig et al. 1998; Mocellin et al. 2004), this regulation of IL 10 clearly demonstrates for the first time that the lung not only responds on a whole organ basis, but also that the response is separate and distinct from the response local to the inflammatory stimulus rather than a diminished version of the innate inflammation stimulated in the challenged segment.

The purpose of the up regulation of an anti-inflammatory cytokine remote from the site of localised inflammation would logically seem to be as a protective measure against wide ranging inflammation throughout the lung and thus preventing organ-wide tissue damage that might lead to organ failure which is the primary cause of death seen in pulmonary ARDS (Dokka et al. 2000; Inoue 2000; Lo, Fu, & Cryer 1998). A logical extrapolation from this observation would be that the progression seen in ARDS is due, at least in part, to the failure of this mechanism, or indeed an overwhelming of the system. This supposition is supported by the evidence that IL 10 has been shown to ameliorate the lung and multi-organ failure associated with ALI/ARDS when given artificially (Lo, Fu, & Cryer 1998).

In order to investigate the nature of the remote response in lung tissue more fully, a sensitive, quantitative platform was needed to probe the molecular profile of the expressed genes in the remote segment. Microarrays have been established as an appropriate and powerful technology for investigating the expression profiles of multiple genes simultaneously. They have been used and their capability established in a number of lung disease models – including asthma (Zimmermann et al. 2003; Zou et al. 2002), acute sepsis (Leikauf et al. 2001) and cancer (Jiang et al. 2004; Hoang et al. 2004; Ikehara et al. 2004) in a variety of comparative species, particularly human (reviewed by Grant (Grant et al. 2004)), mouse (reviewed by Wigle (Wigle, Rossant, & Jurisica 2001)) and rat (reviewed by Schultz (Shultz et al. 2004)) and have been used to investigate LPS induced inflammatory responses both *in vivo* (Leikauf et al. 2001) and *in vitro* (Fessler et al. 2002).

Due to the lack of known and characterised gene sequences in sheep, a combination of suppressive subtractive hybridisation (SSH) library construction (Diatchenko et al. 1996) and homology searching (Altschul et al. 1990) for known sheep homologs

within bovine libraries was used to generate probes for use on a novel ovine lung microarray.

The SSH method generated a library consisting of approximately 1,200 clones. These clones contained sequences representing transcripts either differentially or exclusively expressed in the lung compared with a combined population of brain, heart, muscle, liver and kidney transcripts. Of the 238 clone inserts sequenced, 100 different groups of genes (contigs) were found, indicating that the library is approximately 2.5 fold redundant. If such a relationship holds for the remaining clones, between 400 and 500 ovine genes are represented in this library. This resource is of considerable value and represents a significant avenue for further investigation into both lung biology and sheep genetics. The lack of any large-scale full length genome sequencing program analogous to those for humans, rodent species and cattle (www.genomeonline.com (Bernal, Ear, & Kyrpides 2001; Kyrpides 1999)) adds further weight to the significance of this library as a basis for genetic investigation in sheep. The ability to use the clones available as starting point for characterisation and full length sequencing of large numbers of ovine genes known to be involved in lung biology enables this library to generate considerable knowledge concerning the ovine lung disease model in the future.

There has been no report in the body of available literature of an ovine microarray being used in the past, therefore the new array generated from the SSH library and homology searches represents a unique resource in that regard. Whilst it is unlikely not to be joined by other ovine arrays in the near future no commercial resources are available, or likely to become available, that duplicate this microarray. The ovine lung is highly suited to investigating a variety of pulmonary diseases, and this array is capable of providing considerable molecular analysis for any and all of these model

systems. Indeed this array represents a considerable avenue for molecular studies to characterise these model systems as well as offering the potential for investigations into fundamental ovine lung biology and related comparative respiratory physiology and molecular biology.

The results of the array analysis confirmed the hypothesis that the lung reacts as a whole organ to local inflammatory stimulus. The microarray results clearly demonstrate that the response in the remote segment is complex and different to that seen in the direct segment. The response in the remote segment involves a number of genetic pathways and molecular networks and is a response that has not been reported in the sheep before now. Though the overall cellular and tissue effects, and purpose of these molecular interactions initiated in the remote segment are beyond the scope of this project the data generated provide a unique platform for further study into the remote response to LPS and local sepsis, as well as the fundamental innate response of the lung to direct stimulation with bacterial LPS.

The prevalence of segmental challenge models in all areas of lung disease research (Zangrilli et al. 1995; Koh et al. 1993; Dupuis et al. 1992; O'Grady et al. 2001; Becker et al. 1999), and the reliance of such models on the integrity of the segmental independence of the lung further highlights the importance of these data. Whilst the full characterisation and hypothesis-driven investigation into the specific nature of the remote response remains to be conducted, it is clear that any segmental challenges will need to account for the remote, organ-wide response during selection of appropriate controls in future studies.

A concerning feature of the microarray results was the absence of any significant up regulation of the classical inflammatory markers in the directly challenged lung segment. It is particularly notable that the cytokines that were seen to be up regulated

using the real-time PCR technique were not present in the lists of significantly regulated genes. This statement excludes IL 6 although this cytokine was significantly down regulated on analysis with the microarray, but considerably increased its expression when analysed using real-time PCR technique.

The only cytokine altered in the remote segment by real-time PCR was IL 10 and this, too, was not significantly altered in the microarray analysis. Some possible reasons for these discrepancies are as follows. The length of cDNA used in printing the spots on the microarray, along with the relatively poorly characterised nature of each clone used render this technology susceptible to cross-hybridisation reactions, a problem that does not affect PCR due to the highly specific design of PCR primer pairs, and this cross hybridisation may result in a small minority of elements 'mis-firing' and giving incorrect results. A further consequence of the relatively low specificity of binding of array elements in this system is that the overall signal to which everything is normalised may quench a considerable level of signal strength. It has been established that microarray data does introduce a measurable bias towards underestimation of signal variation between samples when compared with specificity-dependent techniques such as PCR.

The practical and longer-term therapeutic benefits to be derived from the manipulation of the organ wide response to LPS remain to be elucidated. The physiological and phenotypic effects of the response are, as yet, unclear – as is the level to which the impacted networks influence the biology of the organ when a secondary challenge is presented. As has been shown in previous literature, this potential secondary challenge may be the initiating factor and major determinant in the evolution of ARDS and its associated organ failure (Pelosi et al. 2003). Further investigation of this response may indicate the level of challenge at which any

protective, anti-inflammatory remote response may break down and at which the inflammatory impact seen in ARDS becomes evident.

An initial investigation (presented in Appendix V) with limited numbers of animals (n=6) was carried out which showed no significant changes in the numbers of neutrophils infiltrating a contra-lateral segment following local stimulation with LPS and then a repeat challenge in the contra-lateral lung segment. This equivocal result would indicate that the protective response is insufficient to prevent or significantly lower the inflammatory reaction to direct LPS challenge, however greater numbers of replicates and a more detailed time-course analysis study would shed further light on this aspect of the response.

The mechanism by which the lung responds remotely is also not addressed within the scope of this project, and will require considerable work to determine. Previously advanced hypotheses from different model systems concerning bilateral responses have centred around the concept of neurogenic immune regulation (Barnes 1996; Downing & Miyan 2000; Perez Fontan 2002; Tseng et al. 2001) – which has been particularly investigated in arthritis (Lombard et al. 1999; Mapp et al. 1993; Miagkov et al. 1998; Milligan et al. 2003; Scott, Lam, & Ferrell 1994). This area would be one to be considered for any future investigations into the mechanism of remote responses.

Other possible mechanisms include systemic circulation of immune regulators following local LPS instillation. In this regard, interferon (Halloran et al. 1992) has been suggested, though cytokines, chemokines or other immune modulators may also serve as possible signalling molecules. The levels of these mediators are relatively simple to investigate both at the molecular genetic and protein levels, and may

provide an initial point from which to initiate investigations into remote response to local inflammation mechanisms.

Further investigation into these factors would be of considerable value and should include a time-course investigation of both systemic and tissue levels of IL 10, along with pro and other anti-inflammatory regulators and the related profiles of cellular changes. Such a design would potentially facilitate the identification of the point at which the inflammatory modulation in systemic and tissue compartments differs significantly and thereby potentially renders the lung susceptible to progression rather than resolution.

In conclusion it can be clearly stated that the lung responds on a whole organ basis to local LPS-induce innate immune-derived inflammation. Also that the contra-lateral lung initiates a number of complex sub-phenotypic molecular networks following this local inflammatory response.

References

Aderka, D., Le, J. M., & Vilcek, J. 1989, "IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice", *J.Immunol.*, vol. 143, no. 11, pp. 3517-3523.

Ahmad, A., Bayley, D. L., He, S., & Stockley, R. A. 2003, "Myeloid related protein-8/14 stimulates interleukin-8 production in airway epithelial cells", *Am.J.Respir.Cell Mol.Biol.*, vol. 29, no. 4, pp. 523-530.

Ahmed, N. A., McGill, S., Yee, J., Hu, F., Michel, R. P., & Christou, N. V. 1999, "Mechanisms for the diminished neutrophil exudation to secondary inflammatory sites in infected patients with a systemic inflammatory response (sepsis)", *Crit Care Med.*, vol. 27, no. 11, pp. 2459-2468.

Aldridge, A. J. 2002, "Role of the neutrophil in septic shock and the adult respiratory distress syndrome", *Eur.J.Surg.*, vol. 168, no. 4, pp. 204-214.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. 1990, "Basic local alignment search tool", *J.Mol.Biol.*, vol. 215, no. 3, pp. 403-410.

Ando, M., Murakami, Y., Kojima, F., Endo, H., Kitasato, H., Hashimoto, A., Kobayashi, H., Majima, M., Inoue, M., Kondo, H., Kawai, S., & Hayashi, I. 2003, "Retrovirally introduced prostaglandin D2 synthase suppresses lung injury induced by bleomycin", *Am.J.Respir.Cell Mol.Biol.*, vol. 28, no. 5, pp. 582-591.

Appelberg, R. 1994, "Protective role of interferon gamma, tumor necrosis factor alpha and interleukin-6 in Mycobacterium tuberculosis and M. avium infections", *Immunobiology*, vol. 191, no. 4-5, pp. 520-525.

Asnicar, M. A., Henegariu, O., Shaw, M. M., Goheen, M. P., Bartlett, M. S., Smith, J. W., & Lee, C. H. 2001, "Alteration in expression of the rat mitochondrial ATPase 6 gene during Pneumocystis carinii infection", *BMC.Microbiol.*, vol. 1, no. 1, p. 8.

Ault, B. H. & Colten, H. R. 1994, "Cellular specificity of murine renal C3 expression in two models of inflammation", *Immunology*, vol. 81, no. 4, pp. 655-660.

Baeza-Squiban, A., Bonvallot, V., Boland, S., & Marano, F. 1999, "Airborne particles evoke an inflammatory response in human airway epithelium. Activation of transcription factors", *Cell Biol.Toxicol.*, vol. 15, no. 6, pp. 375-380.

Balamugesh, T., Kaur, S., Majumdar, S., & Behera, D. 2003, "Surfactant protein-A levels in patients with acute respiratory distress syndrome", *Indian J.Med.Res.*, vol. 117, pp. 129-133.

Bamburg, J. R. & Wiggan, O. P. 2002, "ADF/cofilin and actin dynamics in disease", *Trends Cell Biol.*, vol. 12, no. 12, pp. 598-605.

Barnes, P. J. 1996, "Neuroeffector mechanisms: the interface between inflammation and neuronal responses", *J.Allergy Clin.Immunol.*, vol. 98, no. 5 Pt 2, p. S73-S81.

Barton, B. E. & Jackson, J. V. 1993, "Protective role of interleukin 6 in the lipopolysaccharide-galactosamine septic shock model", *Infect.Immun.*, vol. 61, no. 4, pp. 1496-1499.

Basoglu, M., Kiziltunc, A., Yildirgan, M. I., Gumustekin, K., Gumus, M., Yildirim, A., & Atamanalp, S. S. 2002, "Recombinant human growth hormone modulates the hepatic acute-phase response and P-selectin in burned rats", *Burns*, vol. 28, no. 8, pp. 760-764.

Baumann, H. & Gauldie, J. 1994, "The acute phase response", *Immunol.Today*, vol. 15, no. 2, pp. 74-80.

Bazel, S., Andrejko, K. M., Chen, J., & Deutschman, C. S. 1999, "Hepatic gene expression and cytokine responses to sterile inflammation: comparison with cecal ligation and puncture sepsis in the rat", *Shock*, vol. 11, no. 5, pp. 347-355.

Becker, S., Clapp, W. A., Quay, J., Frees, K. L., Koren, H. S., & Schwartz, D. A. 1999, "Compartmentalization of the inflammatory response to inhaled grain dust", *Am.J.Respir.Crit Care Med.*, vol. 160, no. 4, pp. 1309-1318.

Belvisi, M. G. 2003, "Sensory nerves and airway inflammation: role of A delta and C-fibres", *Pulm.Pharmacol.Ther.*, vol. 16, no. 1, pp. 1-7.

Benbernou, N., Matsiota-Bernard, P., Jolivet, C., Ougen, P., & Guenounou, M. 1992, "Tumour necrosis factor, IL-1 and IL-6 in bronchoalveolar washings and their in vitro production during Nippostrongylus brasiliensis infection", *Clin.Exp.Immunol.*, vol. 88, no. 2, pp. 264-268.

Benjamini, Y. a. H. Y. 1995, "*Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing*", *Journal of the Royal Statistical Society B*, vol. 57, pp. 289-300.

Bergad, P. L., Schwarzenberg, S. J., Humbert, J. T., Morrison, M., Amarasinghe, S., Towle, H. C., & Berry, S. A. 2000, "Inhibition of growth hormone action in models of inflammation", *Am.J.Physiol Cell Physiol*, vol. 279, no. 6, p. C1906-C1917.

Berghofer-Hochheimer, Y., Zurek, C., Wolfl, S., Hemmerich, P., & Munder, T. 1998, "L7 protein is a coregulator of vitamin D receptor-retinoid X receptor-mediated transactivation", *J.Cell Biochem.*, vol. 69, no. 1, pp. 1-12.

Berman, J. S., Serlin, D., Li, X., Whitley, G., Hayes, J., Rishikof, D. C., Ricupero, D. A., Liaw, L., Goetschkes, M., & O'Regan, A. W. 2004, "Altered bleomycin-induced lung fibrosis in osteopontin-deficient mice", *Am.J.Physiol Lung Cell Mol.Physiol*, vol. 286, no. 6, p. L1311-L1318.

Bernal, A., Ear, U., & Kyrpides, N. 2001, "Genomes OnLine Database (GOLD): a monitor of genome projects world-wide", *Nucleic Acids Res.*, vol. 29, no. 1, pp. 126-127.

Bertram, J., Palfner, K., Hiddemann, W., & Kneba, M. 1998, "Overexpression of ribosomal proteins L4 and L5 and the putative alternative elongation factor PTI-1 in the doxorubicin resistant human colon cancer cell line LoVoDxR", *Eur.J.Cancer*, vol. 34, no. 5, pp. 731-736.

Bevort, M. & Leffers, H. 2000, "Down regulation of ribosomal protein mRNAs during neuronal differentiation of human NTERA2 cells", *Differentiation*, vol. 66, no. 2-3, pp. 81-92.

Beyer-Sehlmeyer, G., Hiddemann, W., Wormann, B., & Bertram, J. 1999, "Suppressive subtractive hybridisation reveals differential expression of serglycin, sorcin, bone marrow proteoglycan and prostate-tumour-inducing gene I (PTI-1) in drug-resistant and sensitive tumour cell lines of haematopoetic origin", *Eur.J.Cancer*, vol. 35, no. 12, pp. 1735-1742.

Bhathena, P. R., Comhair, S. A., Holroyd, K. J., & Erzurum, S. C. 2000, "Interleukin-9 receptor expression in asthmatic airways In vivo", *Lung*, vol. 178, no. 3, pp. 149-160.

Bice, D. E., Jones, S. E., & Muggenburg, B. A. 1993, "Long-term antibody production after lung immunization and challenge: role of lung and lymphoid tissues", *Am.J.Respir.Cell Mol.Biol.*, vol. 8, no. 6, pp. 662-667.

Bice, D. E. & Muggenburg, B. A. 1996, "Pulmonary immune memory: localized production of antibody in the lung after antigen challenge", *Immunology*, vol. 88, no. 2, pp. 191-197.

Bice, D. E., Seagrave, J., & Green, F. H. 2000, "Animal models of asthma: potential usefulness for studying health effects of inhaled particles", *Inhal.Toxicol.*, vol. 12, no. 9, pp. 829-862.

Bishop, J. O. & Smith, G. P. 1974, "The determination of RNA homogeneity by molecular hybridization", *Cell*, vol. 3, no. 4, pp. 341-346.

Blahnik, M. J., Ramanathan, R., Riley, C. R., & Minoo, P. 2001, "Lipopolysaccharide-induced tumor necrosis factor-alpha and IL-10 production by lung macrophages from preterm and term neonates", *Pediatr.Res.*, vol. 50, no. 6, pp. 726-731.

Bleau, A. M., Maurel, P., Pichette, V., Leblond, F., & du, S. P. 2003, "Interleukin-1beta, interleukin-6, tumour necrosis factor-alpha and interferon-gamma released by a viral infection and an aseptic inflammation reduce CYP1A1, 1A2 and 3A6 expression in rabbit hepatocytes", *Eur.J.Pharmacol.*, vol. 473, no. 2-3, pp. 197-206.

Bone, R. C., Balk, R. A., Cerra, F. B., Dellinger, R. P., Fein, A. M., Knaus, W. A., Schein, R. M., & Sibbald, W. J. 1992, "Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine", *Chest*, vol. 101, no. 6, pp. 1644-1655.

Bone, R. C., Sibbald, W. J., & Sprung, C. L. 1992, "The ACCP-SCCM consensus conference on sepsis and organ failure", *Chest*, vol. 101, no. 6, pp. 1481-1483.

Bonig, H., Packeisen, J., Rohne, B., Hempel, L., Hannen, M., Klein-Vehne, A., Burdach, S., & Korholz, D. 1998, "Interaction between interleukin 10 and interleukin 6 in human B-cell differentiation", *Immunol.Invest*, vol. 27, no. 4-5, pp. 267-280.

BORRIE, J. & MITCHELL, R. M. 1960, "The sheep as an experimental animal in surgical science", *Br.J.Surg.*, vol. 47, pp. 435-445.

Bozic, C. R., Lu, B., Hopken, U. E., Gerard, C., & Gerard, N. P. 1996, "Neurogenic amplification of immune complex inflammation", *Science*, vol. 273, no. 5282, pp. 1722-1725.

Brauer, R. B., Gegenfurtner, C., Neumann, B., Stadler, M., Heidecke, C. D., & Holzmann, B. 2000, "Endotoxin-induced lung inflammation is independent of the complement membrane attack complex", *Infect.Immun.*, vol. 68, no. 3, pp. 1626-1632.

Bribes, E., Bourrie, B., & Casellas, P. 2003, "Ligands of the peripheral benzodiazepine receptor have therapeutic effects in pneumopathies in vivo", *Immunol.Lett.*, vol. 88, no. 3, pp. 241-247.

Brigham, K. L. & Staub, N. C. 1998, "Pulmonary oedema and acute lung injury research", *Am.J.Respir.Crit Care Med.*, vol. 157, no. 4 Pt 2, p. S109-S113.

Brigham, K. L., Woolverton, W. C., Blake, L. H., & Staub, N. C. 1974, "Increased sheep lung vascular permeability caused by pseudomonas bacteremia", *J.Clin.Invest*, vol. 54, no. 4, pp. 792-804.

British Thoracic Society 1999, *The Burden of Lung Disease: A Statistics Report from the British Thoracic Society*, British Thoracic Society.

Brogden, K. A., Ackermann, M. R., & Debey, B. M. 1995, "Pasteurella haemolytica lipopolysaccharide-associated protein induces pulmonary inflammation after bronchoscopic deposition in calves and sheep", *Infect.Immun.*, vol. 63, no. 9, pp. 3595-3599.

Brogden, K. A., Cutlip, R. C., & Lehmkuhl, H. D. 1984, "Response of sheep after localized deposition of lipopolysaccharide in the lung", *Exp.Lung Res.*, vol. 7, no. 2, pp. 123-132.

Brunori, M., Giuffre, A., Sarti, P., Stubauer, G., & Wilson, M. T. 1999, "Nitric oxide and cellular respiration", *Cell Mol.Life Sci.*, vol. 56, no. 7-8, pp. 549-557.

Bryant, R. E., Crouse, R., & Deagen, J. T. 2004, "Zinc, iron, copper, selenium, lactoferrin, and ferritin in human pus", *Am.J.Med.Sci.*, vol. 327, no. 2, pp. 73-76.

Bustin, S. A. 2002, "Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems", *J.Mol.Endocrinol.*, vol. 29, no. 1, pp. 23-39.

Bustin, S. A., Li, S. R., Phillips, S., & Dorudi, S. 2001, "Expression of HLA class II in colorectal cancer: evidence for enhanced immunogenicity of microsatellite-instability-positive tumours", *Tumour.Biol.*, vol. 22, no. 5, pp. 294-298.

Callery, M. P., Kamei, T., Mangino, M. J., & Flye, M. W. 1991, "Organ interactions in sepsis. Host defense and the hepatic-pulmonary macrophage axis", *Arch.Surg.*, vol. 126, no. 1, pp. 28-32.

Carcillo, J. A., Doughty, L., Kofos, D., Frye, R. F., Kaplan, S. S., Sasser, H., & Burckart, G. J. 2003, "Cytochrome P450 mediated-drug metabolism is reduced in

children with sepsis-induced multiple organ failure", *Intensive Care Med.*, vol. 29, no. 6, pp. 980-984.

Cardozo, C., Edelman, J., Jagirdar, J., & Lesser, M. 1991, "Lipopolysaccharide-induced pulmonary vascular sequestration of polymorphonuclear leukocytes is complement independent", *Am.Rev.Respir.Dis.*, vol. 144, no. 1, pp. 173-178.

Carvalho, B., Seruca, R., Buys, C. H., & Kok, K. 2002, "Novel expressed sequences obtained by means of a suppression subtractive hybridisation analysis from the 6q21 region that is frequently deleted in gastric cancer", *Eur.J.Cancer*, vol. 38, no. 8, pp. 1126-1132.

Chandel, N., Budinger, G. R., Kemp, R. A., & Schumacker, P. T. 1995, "Inhibition of cytochrome-c oxidase activity during prolonged hypoxia", *Am.J.Physiol*, vol. 268, no. 6 Pt 1, p. L918-L925.

Chen, W., Havell, E. A., Gigliotti, F., & Harmsen, A. G. 1993, "Interleukin-6 production in a murine model of Pneumocystis carinii pneumonia: relation to resistance and inflammatory response", *Infect.Immun.*, vol. 61, no. 1, pp. 97-102.

Chen, Y. H., Layne, M. D., Chung, S. W., Ejima, K., Baron, R. M., Yet, S. F., & Perrella, M. A. 2003, "Elk-3 is a transcriptional repressor of nitric-oxide synthase 2", *J.Biol.Chem.*, vol. 278, no. 41, pp. 39572-39577.

Cheng, K. T., Hou, W. C., Huang, Y. C., & Wang, L. F. 2003, "Baicalin induces differential expression of cytochrome C oxidase in human lung H441 cell", *J.Agric.Food Chem.*, vol. 51, no. 25, pp. 7276-7279.

Chester, K. A., Robson, L., Begent, R. H., Talbot, I. C., Pringle, J. H., Primrose, L., Macpherson, A. J., Boxer, G., Southall, P., & Malcolm, A. D. 1989, "Identification of a human ribosomal protein mRNA with increased expression in colorectal tumours", *Biochim.Biophys.Acta*, vol. 1009, no. 3, pp. 297-300.

Cheung, V. G., Morley, M., Aguilar, F., Massimi, A., Kucherlapati, R., & Childs, G. 1999, "Making and reading microarrays", *Nat.Genet.*, vol. 21, no. 1 Suppl, pp. 15-19.

Chollet-Martin, S., Jourdain, B., Gibert, C., Elbim, C., Chastre, J., & Gougerot-Pocidalo, M. A. 1996, "Interactions between neutrophils and cytokines in blood and alveolar spaces during ARDS", *Am.J.Respir.Crit Care Med.*, vol. 154, no. 3 Pt 1, pp. 594-601.

Cohen, M. V. & Downey, J. M. 1990, "Myocardial stunning in dogs: preconditioning effect and influence of coronary collateral flow", *Am.Heart J.*, vol. 120, no. 2, pp. 282-291.

Collie, D. D., MacAldowie, C. N., Pemberton, A. D., Woodall, C. J., McLean, N., Hodgson, C., Kennedy, M. W., & Miller, H. R. 2001, "Local lung responses following local lung challenge with recombinant lungworm antigen in systemically sensitized sheep", *Clin.Exp.Allergy*, vol. 31, no. 10, pp. 1636-1647.

Colville-Nash, P. R. & Gilroy, D. W. 2001, "Potential adverse effects of cyclooxygenase-2 inhibition: evidence from animal models of inflammation", *BioDrugs.*, vol. 15, no. 1, pp. 1-9.

Cooper, P., Potter, S., Mueck, B., Yousefi, S., & Jarai, G. 2001, "Identification of genes induced by inflammatory cytokines in airway epithelium", *Am.J.Physiol Lung Cell Mol.Physiol*, vol. 280, no. 5, p. L841-L852.

Costa, J. J., Matossian, K., Resnick, M. B., Beil, W. J., Wong, D. T., Gordon, J. R., Dvorak, A. M., Weller, P. F., & Galli, S. J. 1993, "Human eosinophils can express the cytokines tumor necrosis factor-alpha and macrophage inflammatory protein-1 alpha", *J.Clin.Invest*, vol. 91, no. 6, pp. 2673-2684.

Crestani, B. & Aubier, M. 1998, "Inflammatory role of alveolar epithelial cells", *Kidney Int.Suppl*, vol. 65, p. S88-S93.

Crestani, B., Cornillet, P., Dehoux, M., Rolland, C., Guenounou, M., & Aubier, M. 1994, "Alveolar type II epithelial cells produce interleukin-6 in vitro and in vivo. Regulation by alveolar macrophage secretory products", *J.Clin.Invest*, vol. 94, no. 2, pp. 731-740.

Dalrymple, S. A., Lucian, L. A., Slattery, R., McNeil, T., Aud, D. M., Fuchino, S., Lee, F., & Murray, R. 1995, "Interleukin-6-deficient mice are highly susceptible to Listeria monocytogenes infection: correlation with inefficient neutrophilia", *Infect.Immun.*, vol. 63, no. 6, pp. 2262-2268.

de Astorza, B., Cortes, G., Crespi, C., Saus, C., Rojo, J. M., & Alberti, S. 2004, "C3 promotes clearance of Klebsiella pneumoniae by A549 epithelial cells", *Infect.Immun.*, vol. 72, no. 3, pp. 1767-1774.

De, R., V 2002, "Mechanisms and markers of airway inflammation in cystic fibrosis", *Eur.Respir.J.*, vol. 19, no. 2, pp. 333-340.

Decaris, E., Guingamp, C., Chat, M., Philippe, L., Grillasca, J. P., Abid, A., Minn, A., Gillet, P., Netter, P., & Terlain, B. 1999, "Evidence for neurogenic transmission inducing degenerative cartilage damage distant from local inflammation", *Arthritis Rheum.*, vol. 42, no. 9, pp. 1951-1960.

Dehoux, M. S., Boutten, A., Ostinelli, J., Seta, N., Dombret, M. C., Crestani, B., Deschenes, M., Trouillet, J. L., & Aubier, M. 1994, "Compartmentalized cytokine production within the human lung in unilateral pneumonia", *Am.J.Respir.Crit Care Med.*, vol. 150, no. 3, pp. 710-716.

Demetriou, M., Binkert, C., Sukhu, B., Tenenbaum, H. C., & Dennis, J. W. 1996, "Fetuin/alpha2-HS glycoprotein is a transforming growth factor-beta type II receptor mimic and cytokine antagonist", *J.Biol.Chem.*, vol. 271, no. 22, pp. 12755-12761.

Dentener, M. A., Vreugdenhil, A. C., Hoet, P. H., Vernooy, J. H., Nieman, F. H., Heumann, D., Janssen, Y. M., Buurman, W. A., & Wouters, E. F. 2000, "Production of the acute-phase protein lipopolysaccharide-binding protein by respiratory type II epithelial cells: implications for local defense to bacterial endotoxins", *Am.J.Respir.Cell Mol.Biol.*, vol. 23, no. 2, pp. 146-153.

Derfalvi, B., Igaz, P., Fulop, K. A., Szalai, C., & Falus, A. 2000, "Interleukin-6-induced production of type II acute phase proteins and expression of junB gene are downregulated by human recombinant growth hormone in vitro", *Cell Biol.Int.*, vol. 24, no. 2, pp. 109-114.

Diamond, G., Legarda, D., & Ryan, L. K. 2000, "The innate immune response of the respiratory epithelium", *Immunol.Rev.*, vol. 173, pp. 27-38.

Diatchenko, L., Lau, Y. F., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D., & Siebert, P. D. 1996, "Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries", *Proc.Natl.Acad.Sci.U.S.A*, vol. 93, no. 12, pp. 6025-6030.

DiCosmo, B. F., Geba, G. P., Picarella, D., Elias, J. A., Rankin, J. A., Stripp, B. R., Whitsett, J. A., & Flavell, R. A. 1994, "Airway epithelial cell expression of interleukin-6 in transgenic mice. Uncoupling of airway inflammation and bronchial hyperreactivity", *J.Clin.Invest*, vol. 94, no. 5, pp. 2028-2035.

Dinarello, C. A. 1994, "The biological properties of interleukin-1", *Eur.Cytokine Netw.*, vol. 5, no. 6, pp. 517-531.

Dobrovolskaia, M. A. & Vogel, S. N. 2002, "Toll receptors, CD14, and macrophage activation and deactivation by LPS", *Microbes.Infect.*, vol. 4, no. 9, pp. 903-914.

Dokka, S., Malanga, C. J., Shi, X., Chen, F., Castranova, V., & Rojanasakul, Y. 2000, "Inhibition of endotoxin-induced lung inflammation by interleukin-10 gene transfer in mice", *Am.J.Physiol Lung Cell Mol.Physiol*, vol. 279, no. 5, p. L872-L877.

Dougherty, J. D. & Geschwind, D. H. 2002, "Subtraction-coupled Custom Microarray Analysis for Gene Discovery and Gene Expression Studies in the CNS", *Chem.Senses*, vol. 27, no. 3, pp. 293-298.

Downing, J. E. & Miyan, J. A. 2000, "Neural immunoregulation: emerging roles for nerves in immune homeostasis and disease", *Immunol.Today*, vol. 21, no. 6, pp. 281-289.

Drouin, S. M., Corry, D. B., Kildsgaard, J., & Wetsel, R. A. 2001, "Cutting edge: the absence of C3 demonstrates a role for complement in Th2 effector functions in a murine model of pulmonary allergy", *J.Immunol.*, vol. 167, no. 8, pp. 4141-4145.

Duggan, D. J., Bittner, M., Chen, Y., Meltzer, P., & Trent, J. M. 1999, "Expression profiling using cDNA microarrays", *Nat.Genet.*, vol. 21, no. 1 Suppl, pp. 10-14.

Dupuis, R., Collins, D. S., Koh, Y. Y., Pollice, M., Albertine, K. H., Fish, J. E., & Peters, S. P. 1992, "Effect of antigen dose on the recruitment of inflammatory cells to the lung by segmental antigen challenge", *J.Allergy Clin.Immunol.*, vol. 89, no. 4, pp. 850-857.

Eaton, S., Fukumoto, K., Stefanutti, G., Spitz, L., Zammit, V. A., & Pierro, A. 2003, "Myocardial carnitine palmitoyltransferase I as a target for oxidative modification in inflammation and sepsis", *Biochem.Soc.Trans.*, vol. 31, no. Pt 6, pp. 1133-1136.

Eberwine, J. 1996, "Amplification of mRNA populations using aRNA generated from immobilized oligo(dT)-T7 primed cDNA", *Biotechniques*, vol. 20, no. 4, pp. 584-591.

Egan, P. J., Kimpton, W., Seow, H. F., Bowles, V. M., Brandon, M. R., & Nash, A. D. 1996, "Inflammation-induced changes in the phenotype and cytokine profile of cells migrating through skin and afferent lymph", *Immunology*, vol. 89, no. 4, pp. 539-546.

Eissa, N. T. & Erzurum, S. C. 2001, "Flexible bronchoscopy in molecular biology", *Clin.Chest Med.*, vol. 22, no. 2, pp. 343-53, ix.

Endege, W. O., Steinmann, K. E., Boardman, L. A., Thibodeau, S. N., & Schlegel, R. 1999, "Representative cDNA libraries and their utility in gene expression profiling", *Biotechniques*, vol. 26, no. 3, pp. 542-8, 550.

Feingold, K., Kim, M. S., Shigenaga, J., Moser, A., & Grunfeld, C. 2004, "Altered expression of nuclear hormone receptors and coactivators in mouse heart during the acute-phase response", *Am.J.Physiol Endocrinol.Metab*, vol. 286, no. 2, p. E201-E207.

Ferrari, S., Griesenbach, U., Geddes, D. M., & Alton, E. 2003, "Immunological hurdles to lung gene therapy", *Clin.Exp.Immunol.*, vol. 132, no. 1, pp. 1-8.

Fessler, M. B., Malcolm, K. C., Duncan, M. W., & Worthen, G. S. 2002, "A genomic and proteomic analysis of activation of the human neutrophil by lipopolysaccharide and its mediation by p38 mitogen-activated protein kinase", *J.Biol.Chem.*

Finotto, S., Ohno, I., Marshall, J. S., Gauldie, J., Denburg, J. A., Dolovich, J., Clark, D. A., & Jordana, M. 1994, "TNF-alpha production by eosinophils in upper airways inflammation (nasal polyposis)", *J.Immunol.*, vol. 153, no. 5, pp. 2278-2289.

Forsgren, P. E., Modig, J. A., Dahlback, C. M., & Axelsson, B. I. 1990, "Prophylactic treatment with an aerosolized corticosteroid liposome in a porcine model of early ARDS induced by endotoxaemia", *Acta Chir Scand.*, vol. 156, no. 6-7, pp. 423-431.

Francois, E., Wang, D. Y., Fulthorpe, R., Liss, S. N., & Edwards, E. A. 2003, "DNA microarrays for detecting endocrine-disrupting compounds", *Biotechnol.Adv.*, vol. 22, no. 1-2, pp. 17-26.

Frevert, C. W., Warner, A. E., & Kobzik, L. 1994, "Defective pulmonary recruitment of neutrophils in a rat model of endotoxemia", *Am.J.Respir.Cell Mol.Biol.*, vol. 11, no. 6, pp. 716-723.

Frigerio, J. M., Berthezene, P., Garrido, P., Ortiz, E., Barthellemy, S., Vasseur, S., Sastre, B., Seleznieff, I., Dagorn, J. C., & Iovanna, J. L. 1995, "Analysis of 2166 clones from a human colorectal cancer cDNA library by partial sequencing", *Hum.Mol.Genet.*, vol. 4, no. 1, pp. 37-43.

Fujii, T., Hayashi, S., Hogg, J. C., Vincent, R., & Van Eeden, S. F. 2001, "Particulate matter induces cytokine expression in human bronchial epithelial cells", *Am.J.Respir.Cell Mol.Biol.*, vol. 25, no. 3, pp. 265-271.

Gamble, J. R., Harlan, J. M., Klebanoff, S. J., & Vadas, M. A. 1985, "Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor", *Proc.Natl.Acad.Sci.U.S.A*, vol. 82, no. 24, pp. 8667-8671.

George, C. L., White, M. L., O'Neill, M. E., Thorne, P. S., Schwartz, D. A., & Snyder, J. M. 2003, "Altered surfactant protein A gene expression and protein metabolism associated with repeat exposure to inhaled endotoxin", *Am.J.Physiol Lung Cell Mol.Physiol*, vol. 285, no. 6, p. L1337-L1344.

Gilroy, D. W., Colville-Nash, P. R., Willis, D., Chivers, J., Paul-Clark, M. J., & Willoughby, D. A. 1999, "Inducible cyclooxygenase may have anti-inflammatory properties", *Nat.Med.*, vol. 5, no. 6, pp. 698-701.

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., & Mathieu, C. 2001, "An overview of real-time quantitative PCR: applications to quantify cytokine gene expression", *Methods*, vol. 25, no. 4, pp. 386-401.

Gladue, R. P., Laquerre, A. M., Magna, H. A., Carroll, L. A., O'Donnell, M., Changelian, P. S., & Franke, A. E. 1994, "In vivo augmentation of IFN-gamma with a rIL-12 human/mouse chimera: pleiotropic effects against infectious agents in mice and rats", *Cytokine*, vol. 6, no. 3, pp. 318-328.

Glare, E. M., Divjak, M., Bailey, M. J., & Walters, E. H. 2002, "beta-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels", *Thorax*, vol. 57, no. 9, pp. 765-770.

Gohin, I., Olivier, M., Lantier, I., Pepin, M., & Lantier, F. 1997, "Analysis of the immune response in sheep efferent lymph during Salmonella abortusovis infection", *Vet.Immunol.Immunopathol.*, vol. 60, no. 1-2, pp. 111-130.

Goldstone, S. D. & Lavin, M. F. 1993, "Isolation of a cDNA clone, encoding the ribosomal protein S20, downregulated during the onset of apoptosis in a human leukaemic cell line", *Biochem.Biophys.Res.Commun.*, vol. 196, no. 2, pp. 619-623.

Goodman, R. B., Forstrom, J. W., Osborn, S. G., Chi, E. Y., & Martin, T. R. 1991, "Identification of two neutrophil chemotactic peptides produced by porcine alveolar macrophages", *J.Biol.Chem.*, vol. 266, no. 13, pp. 8455-8463.

Gosselin, D., DeSanctis, J., Boule, M., Skamene, E., Matouk, C., & Radzioch, D. 1995, "Role of tumor necrosis factor alpha in innate resistance to mouse pulmonary infection with Pseudomonas aeruginosa", *Infect.Immun.*, vol. 63, no. 9, pp. 3272-3278.

Grabowski, D. T., Deutsch, W. A., Derda, D., & Kelley, M. R. 1991, "Drosophila AP3, a presumptive DNA repair protein, is homologous to human ribosomal associated protein P0", *Nucleic Acids Res.*, vol. 19, no. 15, p. 4297.

Grant, G. M., Fortney, A., Gorreta, F., Estep, M., Del Giacco, L., Van Meter, A., Christensen, A., Appalla, L., Naouar, C., Jamison, C., Al Timimi, A., Donovan, J., Cooper, J., Garrett, C., & Chandhoke, V. 2004, "Microarrays in cancer research", *Anticancer Res.*, vol. 24, no. 2A, pp. 441-448.

Greenberger, M. J., Strieter, R. M., Kunkel, S. L., Danforth, J. M., Goodman, R. E., & Standiford, T. J. 1995, "Neutralization of IL-10 increases survival in a murine model of Klebsiella pneumonia", *J.Immunol.*, vol. 155, no. 2, pp. 722-729.

Hacia, J. G., Fan, J. B., Ryder, O., Jin, L., Edgemon, K., Ghandour, G., Mayer, R. A., Sun, B., Hsie, L., Robbins, C. M., Brody, L. C., Wang, D., Lander, E. S., Lipshutz, R., Fodor, S. P., & Collins, F. S. 1999, "Determination of ancestral alleles for human single-nucleotide polymorphisms using high-density oligonucleotide arrays", *Nat.Genet.*, vol. 22, no. 2, pp. 164-167.

Hack, C. E., De Groot, E. R., Felt-Bersma, R. J., Nuijens, J. H., Strack Van Schijndel, R. J., Eerenberg-Belmer, A. J., Thijs, L. G., & Aarden, L. A. 1989, "Increased plasma levels of interleukin-6 in sepsis", *Blood*, vol. 74, no. 5, pp. 1704-1710.

Hackett, N. R., Heguy, A., Harvey, B. G., O'Connor, T. P., Luettich, K., Flieder, D. B., Kaplan, R., & Crystal, R. G. 2003, "Variability of antioxidant-related gene expression in the airway epithelium of cigarette smokers", *Am.J.Respir.Cell Mol.Biol.*, vol. 29, no. 3 Pt 1, pp. 331-343.

Haddad, e., McCluskie, K., Birrell, M. A., Dabrowski, D., Pecoraro, M., Underwood, S., Chen, B., De Sanctis, G. T., Webber, S. E., Foster, M. L., & Belvisi, M. G. 2002, "Differential effects of ebselen on neutrophil recruitment, chemokine, and inflammatory mediator expression in a rat model of lipopolysaccharide-induced pulmonary inflammation", *J.Immunol.*, vol. 169, no. 2, pp. 974-982.

Halloran, P. F., Autenried, P., Ramassar, V., Urmson, J., & Cockfield, S. 1992, "Local T cell responses induce widespread MHC expression. Evidence that IFN-gamma induces its own expression in remote sites", *J.Immunol.*, vol. 148, no. 12, pp. 3837-3846.

Hanatani, A., Yoshiyama, M., Kim, S., Omura, T., Ikuno, Y., Takeuchi, K., Iwao, H., & Yoshikawa, J. 1998, "Assessment of cardiac function and gene expression at an early phase after myocardial infarction", *Jpn.Heart J.*, vol. 39, no. 3, pp. 375-388.

Hansen, T. K. 2003, "Growth hormone and mannan-binding lectin: emerging evidence for hormonal regulation of humoral innate immunity", *Minerva Endocrinol.*, vol. 28, no. 1, pp. 75-84.

Harada, A., Sekido, N., Akahoshi, T., Wada, T., Mukaida, N., & Matsushima, K. 1994, "Essential involvement of interleukin-8 (IL-8) in acute inflammation", *J.Leukoc.Biol.*, vol. 56, no. 5, pp. 559-564.

Harkin, D. W., Barros D'Sa, A. A., McCallion, K., Hoper, M., & Campbell, F. C. 2002, "Ischemic preconditioning before lower limb ischemia--reperfusion protects against acute lung injury", *J.Vasc.Surg.*, vol. 35, no. 6, pp. 1264-1273.

Harris, H. W., Gosnell, J. E., & Kumwenda, Z. L. 2000, "The lipemia of sepsis: triglyceride-rich lipoproteins as agents of innate immunity", *J.Endotoxin.Res.*, vol. 6, no. 6, pp. 421-430.

Heremans, H., Dillen, C., Groenen, M., Matthys, P., & Billiau, A. 2000, "Role of interferon-gamma and nitric oxide in pulmonary oedema and death induced by lipopolysaccharide", *Am.J.Respir.Crit Care Med.*, vol. 161, no. 1, pp. 110-117.

Heremans, H., Dillen, C., Put, W., Van Damme, J., & Billiau, A. 1992, "Protective effect of anti-interleukin (IL)-6 antibody against endotoxin, associated with paradoxically increased IL-6 levels", *Eur.J.Immunol.*, vol. 22, no. 9, pp. 2395-2401.

Hicks, R. R., Li, C., Zhang, L., Dhillon, H. S., Prasad, M. R., & Seroogy, K. B. 1999, "Alterations in BDNF and trkB mRNA levels in the cerebral cortex following experimental brain trauma in rats", *J.Neurotrauma*, vol. 16, no. 6, pp. 501-510.

Hiemstra, P. S., van Wetering, S., & Stolk, J. 1998, "Neutrophil serine proteinases and defensins in chronic obstructive pulmonary disease: effects on pulmonary epithelium", *Eur.Respir.J.*, vol. 12, no. 5, pp. 1200-1208.

Higuchi, R., Dollinger, G., Walsh, P. S., & Griffith, R. 1992, "Simultaneous amplification and detection of specific DNA sequences", *Biotechnology (N.Y.)*, vol. 10, no. 4, pp. 413-417.

Higuchi, R., Fockler, C., Dollinger, G., & Watson, R. 1993, "Kinetic PCR analysis: real-time monitoring of DNA amplification reactions", *Biotechnology (N.Y.)*, vol. 11, no. 9, pp. 1026-1030.

Hirano, S. 1997, "Quantitative time-course profiles of bronchoalveolar lavage cells following intratracheal instillation of lipopolysaccharide in mice", *Ind.Health*, vol. 35, no. 3, pp. 353-358.

Hoang, C. D., D'Cunha, J., Tawfic, S. H., Gruessner, A. C., Kratzke, R. A., & Maddaus, M. A. 2004, "Expression profiling of non-small cell lung carcinoma identifies metastatic genotypes based on lymph node tumor burden", *J.Thorac.Cardiovasc.Surg.*, vol. 127, no. 5, pp. 1332-1341.

Hoang, C. D., Zhang, J., Payne, R. M., & Apple, F. S. 1997, "Post-infarction left ventricular remodeling induces changes in creatine kinase mRNA and protein subunit levels in porcine myocardium", *Am.J.Pathol.*, vol. 151, no. 1, pp. 257-264.

Hoffman, A. M., Viel, L., Staempfli, H. R., Muckle, C. A., & Yager, J. A. 1993, "Sensitivity and specificity of bronchoalveolar lavage and protected catheter brush methods for isolating bacteria from foals with experimentally induced pneumonia caused by Klebsiella pneumoniae", *Am.J.Vet.Res.*, vol. 54, no. 11, pp. 1803-1807.

Holgate, S. T., Lackie, P. M., Davies, D. E., Roche, W. R., & Walls, A. F. 1999, "The bronchial epithelium as a key regulator of airway inflammation and remodelling in asthma", *Clin.Exp.Allergy*, vol. 29 Suppl 2, pp. 90-95.

Holub, M. & Lawrence, D. A. 2003, "Influence of endotoxin-induced acute lung injury on pulmonary innate and adaptive immunity", *APMIS*, vol. 111, no. 5, pp. 571-580.

Hoshikawa, Y., Nana-Sinkam, P., Moore, M. D., Sotto-Santiago, S., Phang, T., Keith, R. L., Morris, K. G., Kondo, T., Tuder, R. M., Voelkel, N. F., & Geraci, M. W. 2003, "Hypoxia induces different genes in the lungs of rats compared with mice", *Physiol Genomics*, vol. 12, no. 3, pp. 209-219.

Hunt, L. W., Gleich, G. J., Kita, H., Weiler, D. A., Schroeder, D. R., Vuk-Pavlovic, Z., & Sur, S. 2002, "Removal of bronchoalveolar cells augments the late eosinophilic response to segmental allergen challenge", *Clin.Exp.Allergy*, vol. 32, no. 2, pp. 210-216.

Ichikawa, J. K., Norris, A., Bangera, M. G., Geiss, G. K., 't Wout, A. B., Bumgarner, R. E., & Lory, S. 2000, "Interaction of pseudomonas aeruginosa with epithelial cells: identification of differentially regulated genes by expression microarray analysis of human cDNAs", *Proc.Natl.Acad.Sci.U.S.A*, vol. 97, no. 17, pp. 9659-9664.

Ikegami, M., Weaver, T. E., Conkright, J. J., Sly, P. D., Ross, G. F., Whitsett, J. A., & Glasser, S. W. 2002, "Deficiency of SP-B reveals protective role of SP-C during oxygen lung injury", *J.Appl.Physiol*, vol. 92, no. 2, pp. 519-526.

Ikehara, M., Oshita, F., Sekiyama, A., Hamanaka, N., Saito, H., Yamada, K., Noda, K., Kameda, Y., & Miyagi, Y. 2004, "Genome-wide cDNA microarray screening to

correlate gene expression profile with survival in patients with advanced lung cancer", *Oncol.Rep.*, vol. 11, no. 5, pp. 1041-1044.

Inoue, G. 2000, "Effect of interleukin-10 (IL-10) on experimental LPS-induced acute lung injury", *J.Infect.Chemother.*, vol. 6, no. 1, pp. 51-60.

Inoue, K., Takano, H., Yanagisawa, R., Morita, M., Ichinose, T., Sadakane, K., Yoshino, S., Yamaki, K., Kumagai, Y., Uchiyama, K., & Yoshikawa, T. 2003, "Effect of 15-deoxy-delta 12,14-prostaglandin J2 on acute lung injury induced by lipopolysaccharide in mice", *Eur.J.Pharmacol.*, vol. 481, no. 2-3, pp. 261-269.

Ishida, H., Muchamuel, T., Sakaguchi, S., Andrade, S., Menon, S., & Howard, M. 1994, "Continuous administration of anti-interleukin 10 antibodies delays onset of autoimmunity in NZB/W F1 mice", *J.Exp.Med.*, vol. 179, no. 1, pp. 305-310.

Jagielo, P. J., Thorne, P. S., Watt, J. L., Frees, K. L., Quinn, T. J., & Schwartz, D. A. 1996, "Grain dust and endotoxin inhalation challenges produce similar inflammatory responses in normal subjects", *Chest*, vol. 110, no. 1, pp. 263-270.

Jander, S., Schroeter, M., & Stoll, G. 2000, "Role of NMDA receptor signaling in the regulation of inflammatory gene expression after focal brain ischemia", *J.Neuroimmunol.*, vol. 109, no. 2, pp. 181-187.

Janke, B., Dobrindt, U., Hacker, J., & Blum-Oehler, G. 2001, "A subtractive hybridisation analysis of genomic differences between the uropathogenic E. coli strain 536 and the E. coli K-12 strain MG1655", *FEMS Microbiol.Lett.*, vol. 199, no. 1, pp. 61-66.

Janoff, A., Chanana, A. D., Joel, D. D., Susskind, H., Laurent, P., Yu, S. Y., & Dearing, R. 1983, "Evaluation of the urinary desmosine radioimmunoassay as a monitor of lung injury after endobronchial elastase instillation in sheep", *Am.Rev.Respir.Dis.*, vol. 128, no. 3, pp. 545-551.

Jean, D., Rezaiguia-Delclaux, S., Delacourt, C., Leclercq, R., Lafuma, C., Brun-Buisson, C., Harf, A., & Delclaux, C. 1998, "Protective effect of endotoxin instillation on subsequent bacteria-induced acute lung injury in rats", *Am.J.Respir.Crit Care Med.*, vol. 158, no. 6, pp. 1702-1708.

Jiang, H., Deng, Y., Chen, H. S., Tao, L., Sha, Q., Chen, J., Tsai, C. J., & Zhang, S. 2004, "Joint analysis of two microarray gene-expression data sets to select lung adenocarcinoma marker genes", *BMC.Bioinformatics.*, vol. 5, no. 1, p. 81.

Jones, S. E., Davila, D. R., Haley, P. J., & Bice, D. E. 1993, "The effects of age on immune responses in the antigen-instilled dog lung. Antibody responses in the lung and lymphoid tissues following primary and secondary antigen instillation", *Mech.Ageing Dev.*, vol. 68, no. 1-3, pp. 191-207.

Jordan, J. A., Guo, R. F., Yun, E. C., Sarma, V., Warner, R. L., Crouch, L. D., Senaldi, G., Ulich, T. R., & Ward, P. A. 2001, "Role of IL-18 in acute lung inflammation", *J.Immunol.*, vol. 167, no. 12, pp. 7060-7068.

Joseph, P. M., O'Sullivan, B. P., Lapey, A., Dorkin, H., Oren, J., Balfour, R., Perricone, M. A., Rosenberg, M., Wadsworth, S. C., Smith, A. E., St George, J. A., & Meeker, D. P. 2001, "Aerosol and lobar administration of a recombinant adenovirus to individuals with cystic fibrosis. I. Methods, safety, and clinical implications", *Hum.Gene Ther.*, vol. 12, no. 11, pp. 1369-1382.

Jurado, R. L. 1997, "Iron, infections, and anemia of inflammation", *Clin.Infect.Dis.*, vol. 25, no. 4, pp. 888-895.

Karatzas, C. N. & Turner, J. D. 1997, "Toward altering milk composition by genetic manipulation: current status and challenges", *J.Dairy Sci.*, vol. 80, no. 9, pp. 2225-2232.

Katsuma, S., Nishi, K., Tanigawara, K., Ikawa, H., Shiojima, S., Takagaki, K., Kaminishi, Y., Suzuki, Y., Hirasawa, A., Ohgi, T., Yano, J., Murakami, Y., & Tsujimoto, G. 2001, "Molecular monitoring of bleomycin-induced pulmonary fibrosis by cDNA microarray-based gene expression profiling", *Biochem.Biophys.Res.Commun.*, vol. 288, no. 4, pp. 747-751.

Kawabata, D., Tanaka, M., Fujii, T., Umehara, H., Fujita, Y., Yoshifuji, H., Mimori, T., & Ozaki, S. 2004, "Ameliorative effects of follistatin-related protein/TSC-36/FSTL1 on joint inflammation in a mouse model of arthritis", *Arthritis Rheum.*, vol. 50, no. 2, pp. 660-668.

Kelsen, S. G., Mardini, I. A., Zhou, S., Benovic, J. L., & Higgins, N. C. 1992, "A technique to harvest viable tracheobronchial epithelial cells from living human donors", *Am.J.Respir.Cell Mol.Biol.*, vol. 7, no. 1, pp. 66-72.

Keyvani, K., Reinecke, S., Abts, H. F., Paulus, W., & Witte, O. W. 2000, "Suppression of proteasome C2 contralateral to ischemic lesions in rat brain", *Brain Res.*, vol. 858, no. 2, pp. 386-392.

Khair, O. A., Devalia, J. L., Abdelaziz, M. M., Sapsford, R. J., Tarraf, H., & Davies, R. J. 1994, "Effect of Haemophilus influenzae endotoxin on the synthesis of IL-6, IL-

8, TNF-alpha and expression of ICAM-1 in cultured human bronchial epithelial cells", *Eur.Respir.J.*, vol. 7, no. 12, pp. 2109-2116.

Khovidhunkit, W., Kim, M. S., Memon, R. A., Shigenaga, J. K., Moser, A. H., Feingold, K. R., & Grunfeld, C. 2004, "Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host", *J.Lipid Res.*, vol. 45, no. 7, pp. 1169-1196.

Kidd, B. L., Cruwys, S. C., Garrett, N. E., Mapp, P. I., Jolliffe, V. A., & Blake, D. R. 1995, "Neurogenic influences on contralateral responses during experimental rat monoarthritis", *Brain Res.*, vol. 688, no. 1-2, pp. 72-76.

Kim, J., Chubatsu, L. S., Admon, A., Stahl, J., Fellous, R., & Linn, S. 1995, "Implication of mammalian ribosomal protein S3 in the processing of DNA damage", *J.Biol.Chem.*, vol. 270, no. 23, pp. 13620-13629.

Kinouchi, H., Sharp, F. R., Chan, P. H., Koistinaho, J., Sagar, S. M., & Yoshimoto, T. 1994a, "Induction of c-fos, junB, c-jun, and hsp70 mRNA in cortex, thalamus, basal ganglia, and hippocampus following middle cerebral artery occlusion", *J.Cereb.Blood Flow Metab*, vol. 14, no. 5, pp. 808-817.

Kinouchi, H., Sharp, F. R., Chan, P. H., Koistinaho, J., Sagar, S. M., & Yoshimoto, T. 1994b, "Induction of NGFI-A mRNA following middle cerebral artery occlusion in rats: in situ hybridization study", *Neurosci.Lett.*, vol. 171, no. 1-2, pp. 163-166.

Kishimoto, T., Akira, S., & Taga, T. 1992, "Interleukin-6 and its receptor: a paradigm for cytokines", *Science*, vol. 258, no. 5082, pp. 593-597.

Klei, L. R., Lynch, J. M., Barbano, D. M., Oltenacu, P. A., Lednor, A. J., & Bandler, D. K. 1997, "Influence of milking three times a day on milk quality", *J.Dairy Sci.*, vol. 80, no. 3, pp. 427-436.

Kluth, D. C., Ainslie, C. V., Pearce, W. P., Finlay, S., Clarke, D., Anegon, I., & Rees, A. J. 2001, "Macrophages transfected with adenovirus to express IL-4 reduce inflammation in experimental glomerulonephritis", *J.Immunol.*, vol. 166, no. 7, pp. 4728-4736.

Koh, Y. Y., Dupuis, R., Pollice, M., Albertine, K. H., Fish, J. E., & Peters, S. P. 1993, "Neutrophils recruited to the lungs of humans by segmental antigen challenge display a reduced chemotactic response to leukotriene B4", *Am.J.Respir.Cell Mol.Biol.*, vol. 8, no. 5, pp. 493-499.

Kopf, M., Baumann, H., Freer, G., Freudenberg, M., Lamers, M., Kishimoto, T., Zinkernagel, R., Bluethmann, H., & Kohler, G. 1994, "Impaired immune and acute-phase responses in interleukin-6-deficient mice", *Nature*, vol. 368, no. 6469, pp. 339-342.

Kotsimbos, A. T., Humbert, M., Minshall, E., Durham, S., Pfister, R., Menz, G., Tavernier, J., Kay, A. B., & Hamid, Q. 1997, "Upregulation of alpha GM-CSF-receptor in nonatopic asthma but not in atopic asthma", *J.Allergy Clin.Immunol.*, vol. 99, no. 5, pp. 666-672.

Koyama, S., Sato, E., Nomura, H., Kubo, K., Miura, M., Yamashita, T., Nagai, S., & Izumi, T. 2000, "The potential of various lipopolysaccharides to release IL-8 and G-CSF", *Am.J.Physiol Lung Cell Mol.Physiol*, vol. 278, no. 4, p. L658-L666.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., & Muller, W. 1993, "Interleukin-10-deficient mice develop chronic enterocolitis", *Cell*, vol. 75, no. 2, pp. 263-274.

Kumar, R. K. 1995, "Experimental models in pulmonary pathology", *Pathology*, vol. 27, no. 2, pp. 130-132.

Kuo, H. P., Lin, H. C., Hwang, K. H., Wang, C. H., & Lu, L. C. 2000, "Lipopolysaccharide enhances substance P-mediated neutrophil adherence to epithelial cells and cytokine release", *Am.J.Respir.Crit Care Med.*, vol. 162, no. 5, pp. 1891-1897.

Kuroki, Y. & Sano, H. 1999, "Functional roles and structural analysis of lung collectins SP-A and SP-D", *Biol.Neonate*, vol. 76 Suppl 1, pp. 19-21.

Kuwano, K., Nakashima, N., Inoshima, I., Hagimoto, N., Fujita, M., Yoshimi, M., Maeyama, T., Hamada, N., Watanabe, K., & Hara, N. 2003, "Oxidative stress in lung epithelial cells from patients with idiopathic interstitial pneumonias", *Eur.Respir.J.*, vol. 21, no. 2, pp. 232-240.

Kwong, K. Y., Jones, C. A., Cayabyab, R., Lecart, C., Khuu, N., Rhandhawa, I., Hanley, J. M., Ramanathan, R., deLemos, R. A., & Minoo, P. 1998, "The effects of IL-10 on proinflammatory cytokine expression (IL-1beta and IL-8) in hyaline membrane disease (HMD)", *Clin.Immunol.Immunopathol.*, vol. 88, no. 1, pp. 105-113.

Kyrpides, N. C. 1999, "Genomes OnLine Database (GOLD 1.0): a monitor of complete and ongoing genome projects world-wide", *Bioinformatics.*, vol. 15, no. 9, pp. 773-774.

Laichalk, L. L., Kunkel, S. L., Strieter, R. M., Danforth, J. M., Bailie, M. B., & Standiford, T. J. 1996, "Tumor necrosis factor mediates lung antibacterial host defense in murine Klebsiella pneumonia", *Infect.Immun.*, vol. 64, no. 12, pp. 5211-5218.

Lander, E. S. 1999, "Array of hope", *Nat.Genet.*, vol. 21, no. 1 Suppl, pp. 3-4.

Laprise, C., Sladek, R., Ponton, A., Bernier, M. C., Hudson, T. J., & Laviolette, M. 2004, "Functional classes of bronchial mucosa genes that are differentially expressed in asthma", *BMC.Genomics*, vol. 5, no. 1, p. 21.

Larkin, J. E., Frank, B. C., Gaspard, R. M., Duka, I., Gavras, H., & Quackenbush, J. 2004, "Cardiac transcriptional response to acute and chronic angiotensin II treatments", *Physiol Genomics*, vol. 18, no. 2, pp. 152-166.

Lawrence, E., Van Eeden, S., English, D., & Hogg, J. C. 1996, "Polymorphonuclear leukocyte (PMN) migration in streptococcal pneumonia: comparison of older PMN with those recently released from the marrow", *Am.J.Respir.Cell Mol.Biol.*, vol. 14, no. 3, pp. 217-224.

Le Roux, Y., Laurent, F., & Moussaoui, F. 2003, "Polymorphonuclear proteolytic activity and milk composition change", *Vet.Res.*, vol. 34, no. 5, pp. 629-645.

Le, J. & Vilcek, J. 1987, "Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities", *Lab Invest*, vol. 56, no. 3, pp. 234-248.

Legastelois, I., Greenland, T., Arnaud, P., Mornex, J. F., & Cordier, G. 1994, "Sequencing of the ovine interleukin-8-encoding cDNA using the polymerase chain reaction", *Gene*, vol. 150, no. 2, pp. 367-369.

Leikauf, G. D., McDowell, S. A., Wesselkamper, S. C., Miller, C. R., Hardie, W. D., Gammon, K., Biswas, P. P., Korfhagen, T. R., Bachurski, C. J., Wiest, J. S., Willeke, K., Bingham, E., Leikauf, J. E., Aronow, B. J., & Prows, D. R. 2001, "Pathogenomic mechanisms for particulate matter induction of acute lung injury and inflammation in mice", *Res.Rep.Health Eff.Inst.* no. 105, pp. 5-58.

Lekanne Deprez, R. H., Fijnvandraat, A. C., Ruijter, J. M., & Moorman, A. F. 2002, "Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions", *Anal.Biochem.*, vol. 307, no. 1, pp. 63-69.

Leth-Larsen, R., Nordenbaek, C., Tornoe, I., Moeller, V., Schlosser, A., Koch, C., Teisner, B., Junker, P., & Holmskov, U. 2003, "Surfactant protein D (SP-D) serum

levels in patients with community-acquired pneumonia small star, filled", *Clin.Immunol.*, vol. 108, no. 1, pp. 29-37.

Levine, S. J. 1995, "Bronchial epithelial cell-cytokine interactions in airway inflammation", *J.Investig.Med.*, vol. 43, no. 3, pp. 241-249.

Lim, M. J., Chiang, E. T., Hechtman, H. B., & Shepro, D. 2001, "Inflammation-induced subcellular redistribution of VE-cadherin, actin, and gamma-catenin in cultured human lung microvessel endothelial cells", *Microvasc.Res.*, vol. 62, no. 3, pp. 366-382.

Lin, G., Pearson, A. E., Scamurra, R. W., Zhou, Y., Baarsch, M. J., Weiss, D. J., & Murtaugh, M. P. 1994, "Regulation of interleukin-8 expression in porcine alveolar macrophages by bacterial lipopolysaccharide", *J.Biol.Chem.*, vol. 269, no. 1, pp. 77-85.

Liu, J., Lei, D., Waalkes, M. P., Beliles, R. P., & Morgan, D. L. 2003, "Genomic analysis of the rat lung following elemental mercury vapor exposure", *Toxicol.Sci.*, vol. 74, no. 1, pp. 174-181.

Liu, J. S., Chang, Y. Y., Wu, H. S., Huang, C. Y., Chen, W. H., Lan, M. Y., Kao, Y. F., & Chen, S. S. 2000, "Transtentorial cerebellar c-jun expression after focal cerebral cortical injury in mice", *Neurosci.Lett.*, vol. 282, no. 1-2, pp. 85-88.

Lo, C. J., Fu, M., & Cryer, H. G. 1998, "Interleukin 10 inhibits alveolar macrophage production of inflammatory mediators involved in adult respiratory distress syndrome", *J.Surg.Res.*, vol. 79, no. 2, pp. 179-184.

Loennechen, J. P., Stoylen, A., Beisvag, V., Wisloff, U., & Ellingsen, O. 2001, "Regional expression of endothelin-1, ANP, IGF-1, and LV wall stress in the infarcted rat heart", *Am.J.Physiol Heart Circ.Physiol*, vol. 280, no. 6, p. H2902-H2910.

Lombard, M. C., Weil-Fugazza, J., Ries, C., & Allard, M. 1999, "Unilateral joint inflammation induces bilateral and time-dependent changes in neuropeptide FF binding in the superficial dorsal horn of the rat spinal cord: implication of supraspinal descending systems", *Brain Res.*, vol. 816, no. 2, pp. 598-608.

Mager, W. H. 1988, "Control of ribosomal protein gene expression", *Biochim.Biophys.Acta*, vol. 949, no. 1, pp. 1-15.

Malcolm, K. C., Arndt, P. G., Manos, E. J., Jones, D. A., & Worthen, G. S. 2003, "Microarray analysis of lipopolysaccharide-treated human neutrophils", *Am.J.Physiol Lung Cell Mol.Physiol*, vol. 284, no. 4, p. L663-L670.

Mantovani, A., Muzio, M., Garlanda, C., Sozzani, S., & Allavena, P. 2001, "Macrophage control of inflammation: negative pathways of regulation of inflammatory cytokines", *Novartis.Found.Symp.*, vol. 234, pp. 120-131.

Mapp, P. I., Terenghi, G., Walsh, D. A., Chen, S. T., Cruwys, S. C., Garrett, N., Kidd, B. L., Polak, J. M., & Blake, D. R. 1993, "Monoarthritis in the rat knee induces bilateral and time-dependent changes in substance P and calcitonin gene-related peptide immunoreactivity in the spinal cord", *Neuroscience*, vol. 57, no. 4, pp. 1091-1096.

Mariassy, A. T., McCray, M. N., Lauredo, I. T., Abraham, W. M., & Wanner, A. 1989, "Lectin-detectable effects of localized pneumonia on airway mucous cell populations: role of cyclooxygenase metabolites", *Exp.Lung Res.*, vol. 15, no. 1, pp. 113-137.

Mariassy, A. T. & Plopper, C. G. 1983, "Tracheobronchial epithelium of the sheep: I. Quantitative light-microscopic study of epithelial cell abundance, and distribution", *Anat.Rec.*, vol. 205, no. 3, pp. 263-275.

Mariassy, A. T., Plopper, C. G., St George, J. A., & Wilson, D. W. 1988a, "Tracheobronchial epithelium of the sheep: IV. Lectin histochemical characterization of secretory epithelial cells", *Anat.Rec.*, vol. 222, no. 1, pp. 49-59.

Mariassy, A. T., St George, J. A., Nishio, S. J., & Plopper, C. G. 1988b, "Tracheobronchial epithelium of the sheep: III. Carbohydrate histochemical and cytochemical characterization of secretory epithelial cells", *Anat.Rec.*, vol. 221, no. 1, pp. 540-549.

Martin, T. R. 2000, "Recognition of bacterial endotoxin in the lungs", *Am.J.Respir.Cell Mol.Biol.*, vol. 23, no. 2, pp. 128-132.

Matthay, M. A., Uchida, T., & Fang, X. 2002, "Clinical Acute Lung Injury and Acute Respiratory Distress Syndrome", vol. 4, no. 2, pp. 139-149.

Mazuruk, K., Schoen, T. J., Chader, G. J., Iwata, T., & Rodriguez, I. R. 1996, "Structural organization and chromosomal localization of the human ribosomal protein L9 gene", *Biochim.Biophys.Acta*, vol. 1305, no. 3, pp. 151-162.

McClenahan, D. J., Evanson, O. A., Walcheck, B. K., & Weiss, D. J. 2000, "Association among filamentous actin content, CD11b expression, and membrane deformability in stimulated and unstimulated bovine neutrophils", *Am.J.Vet.Res.*, vol. 61, no. 4, pp. 380-386.

McLaughlin, C. L., Hedrick, H. B., Veenhuizen, J. J., Hintz, R. L., Munyakazi, L., Kasser, T. R., & Baile, C. A. 1994, "Performance, clinical chemistry, and carcass responses of finishing lambs to formulated sometribove (methionyl bovine somatotropin)", *J.Anim Sci.*, vol. 72, no. 10, pp. 2544-2551.

McNeil, J. S., Torrington, K. G., Mundie, T. G., Banks, R. A., Phillips, Y. Y., & Ripple, G. R. 1991, "Prediction of carbon monoxide diffusing capacity of the lung in splenectomized sheep", *Lab Anim Sci.*, vol. 41, no. 1, pp. 63-65.

McNeil, J. S., Torrington, K. G., Mundie, T. G., Ripple, G. R., & Phillips, Y. Y. 1989, "A steady state method of measuring carbon monoxide diffusing capacity of the lung of sheep", *Lab Anim Sci.*, vol. 39, no. 1, pp. 33-36.

Miagkov, A. V., Kovalenko, D. V., Brown, C. E., Didsbury, J. R., Cogswell, J. P., Stimpson, S. A., Baldwin, A. S., & Makarov, S. S. 1998, "NF-kappaB activation provides the potential link between inflammation and hyperplasia in the arthritic joint", *Proc.Natl.Acad.Sci.U.S.A*, vol. 95, no. 23, pp. 13859-13864.

Mikawa, K., Nishina, K., Takao, Y., & Obara, H. 2004, "Intratracheal application of recombinant surfactant protein-C surfactant to rabbits attenuates acute lung injury induced by intratracheal acidified infant formula", *Anesth.Analg.*, vol. 98, no. 5, pp. 1273-9, table.

Miller, C. L., Diglisic, S., Leister, F., Webster, M., & Yolken, R. H. 2004, "Evaluating RNA status for RT-PCR in extracts of postmortem human brain tissue", *Biotechniques*, vol. 36, no. 4, pp. 628-633.

Milligan, E. D., Twining, C., Chacur, M., Biedenkapp, J., O'Connor, K., Poole, S., Tracey, K., Martin, D., Maier, S. F., & Watkins, L. R. 2003, "Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats", *J.Neurosci.*, vol. 23, no. 3, pp. 1026-1040.

Millo, J. L., Schultz, M. J., Williams, C., Weverling, G. J., Ringrose, T., Mackinlay, C. I., van der, P. T., & Garrard, C. S. 2004, "Compartmentalisation of cytokines and cytokine inhibitors in ventilator-associated pneumonia", *Intensive Care Med.*, vol. 30, no. 1, pp. 68-74.

Ming, W. J., Bersani, L., & Mantovani, A. 1987, "Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes", *J.Immunol.*, vol. 138, no. 5, pp. 1469-1474.

Mishra, A. & Rothenberg, M. E. 2003, "Intratracheal IL-13 induces eosinophilic esophagitis by an IL-5, eotaxin-1, and STAT6-dependent mechanism", *Gastroenterology*, vol. 125, no. 5, pp. 1419-1427.

Mitchell, D. M., Emerson, C. J., Collyer, J., & Collins, J. V. 1980a, "Fibreoptic bronchoscopy: ten years on", *Br.Med.J.*, vol. 281, no. 6236, pp. 360-363.

Mitchell, D. M., Mitchell, D. N., Collins, J. V., & Emerson, C. J. 1980b, "Transbronchial lung biopsy through fibreoptic bronchoscope in diagnosis of sarcoidosis", *Br.Med.J.*, vol. 280, no. 6215, pp. 679-681.

Miyazaki, H., Broaddus, V. C., Wiener-Kronish, J. P., Sawa, T., Pittet, J. F., Kravchenko, V., Mathison, J. C., Nishizawa, H., Hattori, S., Yamakawa, T., Yamada, H., & Kudoh, I. 1999, "The effects of two antiinflammatory pretreatments on bacterial-induced lung injury", *Anesthesiology*, vol. 90, no. 6, pp. 1650-1662.

Mocellin, S., Marincola, F., Rossi, C. R., Nitti, D., & Lise, M. 2004, "The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle", *Cytokine Growth Factor Rev.*, vol. 15, no. 1, pp. 61-76.

Monshouwer, M., Witkamp, R. F., Nijmeijer, S. M., Van Leengoed, L. A., Vernooy, H. C., Verheijden, J. H., & Van Miert, A. S. 1996, "A lipopolysaccharide-induced acute phase response in the pig is associated with a decrease in hepatic cytochrome P450-mediated drug metabolism", *J.Vet.Pharmacol.Ther.*, vol. 19, no. 5, pp. 382-388.

Monton, C. & Torres, A. 1998, "Lung inflammatory response in pneumonia", *Monaldi Arch.Chest Dis.*, vol. 53, no. 1, pp. 56-63.

Monton, C., Torres, A., El Ebiary, M., Filella, X., Xaubet, A., & de la Bellacasa, J. P. 1999, "Cytokine expression in severe pneumonia: a bronchoalveolar lavage study", *Crit Care Med.*, vol. 27, no. 9, pp. 1745-1753.

Moore, T. A. & Standiford, T. J. 1998, "The role of cytokines in bacterial pneumonia: an inflammatory balancing act", *Proc.Assoc.Am.Physicians*, vol. 110, no. 4, pp. 297-305.

Muehlstedt, S. G., Lyte, M., & Rodriguez, J. L. 2002, "Increased IL-10 production and HLA-DR suppression in the lungs of injured patients precede the development of nosocomial pneumonia", *Shock*, vol. 17, no. 6, pp. 443-450.

Mueller, G. A., Quinlan, W. M., Doyle, N. A., & Doerschuk, C. M. 1994, "The role of cytoskeletal proteins in neutrophil emigration during pneumonia in rabbits", *Am.J.Respir.Crit Care Med.*, vol. 150, no. 2, pp. 455-461.

Mueller, O., Hahnenberger, K., Dittmann, M., Yee, H., Dubrow, R., Nagle, R., & Ilsley, D. 2000, "A microfluidic system for high-speed reproducible DNA sizing and quantitation", *Electrophoresis*, vol. 21, no. 1, pp. 128-134.

Mueller-Ortiz, S. L., Drouin, S. M., & Wetsel, R. A. 2004, "The alternative activation pathway and complement component C3 are critical for a protective immune response against Pseudomonas aeruginosa in a murine model of pneumonia", *Infect.Immun.*, vol. 72, no. 5, pp. 2899-2906.

Muhlebach, M. S., Reed, W., & Noah, T. L. 2004, "Quantitative cytokine gene expression in CF airway", *Pediatr.Pulmonol.*, vol. 37, no. 5, pp. 393-399.

Muller, E. 1992, "Searching for the cause of the adult respiratory distress syndrome (ARDS)", *Int.J.Artif.Organs*, vol. 15, no. 4, pp. 197-199.

Mullner, N., Lazar, A., & Hrabak, A. 2002, "Enhanced utilization and altered metabolism of arginine in inflammatory macrophages caused by raised nitric oxide synthesis", *Int.J.Biochem.Cell Biol.*, vol. 34, no. 9, pp. 1080-1090.

Mundie, T. G., Januszkiewicz, A. J., Rayburn, D. B., Martin, D. G., & Ripple, G. R. 1991, "Effects of conditioning and maximal incremental exercise on oxygen consumption in sheep", *Am.J.Vet.Res.*, vol. 52, no. 7, pp. 1019-1023.

Munford, R. S. & Pugin, J. 2001, "Normal responses to injury prevent systemic inflammation and can be immunosuppressive", *Am.J.Respir.Crit Care Med.*, vol. 163, no. 2, pp. 316-321.

Munir, S., Singh, S., Kaur, K., & Kapur, V. 2004, "Suppression subtractive hybridization coupled with microarray analysis to examine differential expression of genes in virus infected cells", *Biol.Proced.Online.*, vol. 6, pp. 94-104.

Murtaugh, M. P., Baarsch, M. J., Zhou, Y., Scamurra, R. W., & Lin, G. 1996, "Inflammatory cytokines in animal health and disease", *Vet.Immunol.Immunopathol.*, vol. 54, no. 1-4, pp. 45-55.

Naora, H., Takai, I., Adachi, M., & Naora, H. 1998, "Altered cellular responses by varying expression of a ribosomal protein gene: sequential coordination of enhancement and suppression of ribosomal protein S3a gene expression induces apoptosis", *J.Cell Biol.*, vol. 141, no. 3, pp. 741-753.

Neilsen, P. O., Zimmerman, G. A., & McIntyre, T. M. 2001, "Escherichia coli Braun lipoprotein induces a lipopolysaccharide-like endotoxic response from primary human endothelial cells", *J.Immunol.*, vol. 167, no. 9, pp. 5231-5239.

Neumann, F. & Krawinkel, U. 1997, "Constitutive expression of human ribosomal protein L7 arrests the cell cycle in G1 and induces apoptosis in Jurkat T-lymphoma cells", *Exp.Cell Res.*, vol. 230, no. 2, pp. 252-261.

Neumann, N. F. & Galvez, F. 2002, "DNA microarrays and toxicogenomics: applications for ecotoxicology?", *Biotechnol.Adv.*, vol. 20, no. 5-6, pp. 391-419.

Nohynek, H., Teppo, A. M., Laine, E., Leinonen, M., & Eskola, J. 1991, "Serum tumor necrosis factor-alpha concentrations in children hospitalized for acute lower respiratory tract infection", *J.Infect.Dis.*, vol. 163, no. 5, pp. 1029-1032.

O'Grady, N. P., Preas, H. L., Pugin, J., Fiuza, C., Tropea, M., Reda, D., Banks, S. M., & Suffredini, A. F. 2001, "Local inflammatory responses following bronchial endotoxin instillation in humans", *Am.J.Respir.Crit Care Med.*, vol. 163, no. 7, pp. 1591-1598.

O'Regan, A. 2003, "The role of osteopontin in lung disease", *Cytokine Growth Factor Rev.*, vol. 14, no. 6, pp. 479-488.

Omura, T., Yoshiyama, M., Takeuchi, K., Hanatani, A., Kim, S., Yoshida, K., Izumi, Y., Iwao, H., & Yoshikawa, J. 2000, "Differences in time course of myocardial mRNA expression in non-infarcted myocardium after myocardial infarction", *Basic Res.Cardiol.*, vol. 95, no. 4, pp. 316-323.

Ortiz, L. A., Gambelli, F., McBride, C., Gaupp, D., Baddoo, M., Kaminski, N., & Phinney, D. G. 2003, "Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects", *Proc.Natl.Acad.Sci.U.S.A*, vol. 100, no. 14, pp. 8407-8411.

Otsuka, M., Kato, M., Yoshikawa, T., Chen, H., Brown, E. J., Masuho, Y., Omata, M., & Seki, N. 2001, "Differential expression of the L-plastin gene in human colorectal cancer progression and metastasis", *Biochem.Biophys.Res.Commun.*, vol. 289, no. 4, pp. 876-881.

Out, T. A., Wang, S. Z., Rudolph, K., & Bice, D. E. 2002, "Local T-cell activation after segmental allergen challenge in the lungs of allergic dogs", *Immunology*, vol. 105, no. 4, pp. 499-508.

Oyesiku, N. M., Evans, C. O., Houston, S., Darrell, R. S., Smith, J. S., Fulop, Z. L., Dixon, C. E., & Stein, D. G. 1999, "Regional changes in the expression of neurotrophic factors and their receptors following acute traumatic brain injury in the adult rat brain", *Brain Res.*, vol. 833, no. 2, pp. 161-172.

Palmberg, L., Larsson, B. M., Malmberg, P., & Larsson, K. 1998, "Induction of IL-8 production in human alveolar macrophages and human bronchial epithelial cells in vitro by swine dust", *Thorax*, vol. 53, no. 4, pp. 260-264.

Pavia-Ruz, N., Ortega-Barria, E., Alroy, J., & Pereira, M. E. 1994, "A novel in situ model to study Pneumocystis carinii adhesion to lung alveolar epithelial cells", *J.Immunol.Methods*, vol. 167, no. 1-2, pp. 161-171.

Pechkovsky, D. V., Zissel, G., Ziegenhagen, M. W., Einhaus, M., Taube, C., Rabe, K. F., Magnussen, H., Papadopoulos, T., Schlaak, M., & Muller-Quernheim, J. 2000, "Effect of proinflammatory cytokines on interleukin-8 mRNA expression and protein production by isolated human alveolar epithelial cells type II in primary culture", *Eur.Cytokine Netw.*, vol. 11, no. 4, pp. 618-625.

Pelosi, P., D'Onofrio, D., Chiumello, D., Paolo, S., Chiara, G., Capelozzi, V. L., Barbas, C. S., Chiaranda, M., & Gattinoni, L. 2003, "Pulmonary and extrapulmonary acute respiratory distress syndrome are different", *Eur.Respir.J.Suppl*, vol. 42, pp. 48s-56s.

Penn, R. B., Shaver, J. R., Zangrilli, J. G., Pollice, M., Fish, J. E., Peters, S. P., & Benovic, J. L. 1996, "Effects of inflammation and acute beta-agonist inhalation on beta 2-AR signaling in human airways", *Am.J.Physiol*, vol. 271, no. 4 Pt 1, p. L601-L608.

Peralta, C., Fernandez, L., Panes, J., Prats, N., Sans, M., Pique, J. M., Gelpi, E., & Rosello-Catafau, J. 2001, "Preconditioning protects against systemic disorders associated with hepatic ischemia-reperfusion through blockade of tumor necrosis factor-induced P-selectin up-regulation in the rat", *Hepatology*, vol. 33, no. 1, pp. 100-113.

Perez Fontan, J. J. 2002, "On lung nerves and neurogenic injury", *Ann.Med.*, vol. 34, no. 4, pp. 226-240.

Peterson, B. T., Collins, M. L., Gray, L. D., & Azghani, A. O. 1992, "Aerosolized Pseudomonas elastase and lung fluid balance in anesthetized sheep", *J.Appl.Physiol*, vol. 72, no. 5, pp. 1927-1933.

Pfaffl, M. W., Horgan, G. W., & Dempfle, L. 2002, "Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR", *Nucleic Acids Res.*, vol. 30, no. 9, p. e36.

Pishko, E. J., Kirimanjeswara, G. S., Pilione, M. R., Gopinathan, L., Kennett, M. J., & Harvill, E. T. 2004, "Antibody-mediated bacterial clearance from the lower respiratory tract of mice requires complement component C3", *Eur.J.Immunol.*, vol. 34, no. 1, pp. 184-193.

Plopper, C. G., Mariassy, A. T., & Hill, L. H. 1980, "Ultrastructure of the nonciliated bronchiolar epithelial (Clara) cell of mammalian lung: II. A comparison of horse, steer, sheep, dog, and cat", *Exp.Lung Res.*, vol. 1, no. 2, pp. 155-169.

Pober, J. S., Gimbrone, M. A., Jr., Lapierre, L. A., Mendrick, D. L., Fiers, W., Rothlein, R., & Springer, T. A. 1986, "Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon", *J.Immunol.*, vol. 137, no. 6, pp. 1893-1896.

Pompe, J. C., Kesecioglu, J., Bruining, H. A., & Ince, C. 1996, "Nebulization of endotoxin during mechanical ventilation: an experimental model of ARDS in pigs", *Adv.Exp.Med.Biol.*, vol. 388, pp. 599-602.

Prince, L. S., Karp, P. H., Moninger, T. O., & Welsh, M. J. 2001, "KGF alters gene expression in human airway epithelia: potential regulation of the inflammatory response", *Physiol Genomics*, vol. 6, no. 2, pp. 81-89.

Przyklenk, K., Darling, C. E., Dickson, E. W., & Whittaker, P. 2003, "Cardioprotection 'outside the box'--the evolving paradigm of remote preconditioning", *Basic Res.Cardiol.*, vol. 98, no. 3, pp. 149-157.

Quinn, T. J., Taylor, S., Wohlford-Lenane, C. L., & Schwartz, D. A. 2000, "IL-10 reduces grain dust-induced airway inflammation and airway hyperreactivity", *J.Appl.Physiol*, vol. 88, no. 1, pp. 173-179.

Raasi, S., Schmidtke, G., de Giuli, R., & Groettrup, M. 1999, "A ubiquitin-like protein which is synergistically inducible by interferon-gamma and tumor necrosis factor-alpha", *Eur.J.Immunol.*, vol. 29, no. 12, pp. 4030-4036.

Rabinovici, R., Yeh, C. G., Hillegass, L. M., Griswold, D. E., DiMartino, M. J., Vernick, J., Fong, K. L., & Feuerstein, G. 1992, "Role of complement in endotoxin/platelet-activating factor-induced lung injury", *J.Immunol.*, vol. 149, no. 5, pp. 1744-1750.

Rawn, J., DeCamp, M. M., Jr., Swanson, S. J., Warner, A., Warren, H., & Mentzer, S. J. 2000, "Angiocentric recruitment of lymphocytes into the lung after the intrabronchial instillation of antigen", *Exp.Lung Res.*, vol. 26, no. 2, pp. 89-103.

Raychaudhuri, B., Fisher, C. J., Farver, C. F., Malur, A., Drazba, J., Kavuru, M. S., & Thomassen, M. J. 2000, "Interleukin 10 (IL-10)-mediated inhibition of inflammatory cytokine production by human alveolar macrophages", *Cytokine*, vol. 12, no. 9, pp. 1348-1355.

Reddy, R. C., Chen, G. H., Newstead, M. W., Moore, T., Zeng, X., Tateda, K., & Standiford, T. J. 2001, "Alveolar macrophage deactivation in murine septic peritonitis: role of interleukin 10", *Infect.Immun.*, vol. 69, no. 3, pp. 1394-1401.

Redl, H., Bahrami, S., Schlag, G., & Traber, D. L. 1993, "Clinical detection of LPS and animal models of endotoxemia", *Immunobiology*, vol. 187, no. 3-5, pp. 330-345.

Rehm, S. R., Gross, G. N., & Pierce, A. K. 1980, "Early bacterial clearance from murine lungs. Species-dependent phagocyte response", *J.Clin.Invest*, vol. 66, no. 2, pp. 194-199.

Reid, D. W., Withers, N. J., Francis, L., Wilson, J. W., & Kotsimbos, T. C. 2002, "Iron deficiency in cystic fibrosis: relationship to lung disease severity and chronic Pseudomonas aeruginosa infection", *Chest*, vol. 121, no. 1, pp. 48-54.

Reiner, A., Yekutieli, D., & Benjamini, Y. 2003, "Identifying differentially expressed genes using false discovery rate controlling procedures", *Bioinformatics.*, vol. 19, no. 3, pp. 368-375.

Remick, D. G., Strieter, R. M., Eskandari, M. K., Nguyen, D. T., Genord, M. A., Raiford, C. L., & Kunkel, S. L. 1990, "Role of tumor necrosis factor-alpha in lipopolysaccharide-induced pathologic alterations", *Am.J.Pathol.*, vol. 136, no. 1, pp. 49-60.

Rennard, S. I. 1999, "Inflammation and repair processes in chronic obstructive pulmonary disease", *Am.J.Respir.Crit Care Med.*, vol. 160, no. 5 Pt 2, p. S12-S16.

Reynolds, P. N., Scicchitano, R., & Holmes, M. D. 2001, "Pre-protachykinin-A mRNA is increased in the airway epithelium of smokers with chronic bronchitis", *Respirology.*, vol. 6, no. 3, pp. 187-197.

Riise, G. C., Andersson, B., Ahlstedt, S., Enander, I., Soderberg, M., Lowhagen, O., & Larsson, S. 1996, "Bronchial brush biopsies for studies of epithelial inflammation

in stable asthma and nonobstructive chronic bronchitis", *Eur.Respir.J.*, vol. 9, no. 8, pp. 1665-1671.

Rijneveld, A. W., Lauw, F. N., Schultz, M. J., Florquin, S., Te Velde, A. A., Speelman, P., Van Deventer, S. J., & van der, P. T. 2002, "The role of interferon-gamma in murine pneumococcal pneumonia", *J.Infect.Dis.*, vol. 185, no. 1, pp. 91-97.

Rochemonteix-Galve, B., Marchat-Amoruso, B., Dayer, J. M., & Rylander, R. 1991, "Tumor necrosis factor and interleukin-1 activities in free lung cells after single and repeated inhalation of bacterial endotoxin", *Infect.Immun.*, vol. 59, no. 10, pp. 3646-3650.

Sairanen, T., Ristimaki, A., Karjalainen-Lindsberg, M. L., Paetau, A., Kaste, M., & Lindsberg, P. J. 1998, "Cyclooxygenase-2 is induced globally in infarcted human brain", *Ann.Neurol.*, vol. 43, no. 6, pp. 738-747.

Saitoh, T., Kokue, E., & Shimoda, M. 1999, "The suppressive effects of lipopolysaccharide-induced acute phase response on hepatic cytochrome P450-dependent drug metabolism in rabbits", *J.Vet.Pharmacol.Ther.*, vol. 22, no. 2, pp. 87-95.

Sato, S., Ouellet, N., Pelletier, I., Simard, M., Rancourt, A., & Bergeron, M. G. 2002, "Role of galectin-3 as an adhesion molecule for neutrophil extravasation during streptococcal pneumonia", *J.Immunol.*, vol. 168, no. 4, pp. 1813-1822.

Sato, Y., Walley, K. R., Klut, M. E., English, D., D'Yachkova, Y., Hogg, J. C., & Van Eeden, S. F. 1999, "Nitric oxide reduces the sequestration of polymorphonuclear leukocytes in lung by changing deformability and CD18 expression", *Am.J.Respir.Crit Care Med.*, vol. 159, no. 5 Pt 1, pp. 1469-1476.

Schena, M., Shalon, D., Davis, R. W., & Brown, P. O. 1995, "Quantitative monitoring of gene expression patterns with a complementary DNA microarray", *Science*, vol. 270, no. 5235, pp. 467-470.

Schindler, R., Mancilla, J., Endres, S., Ghorbani, R., Clark, S. C., & Dinarello, C. A. 1990, "Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF", *Blood*, vol. 75, no. 1, pp. 40-47.

Schmeck, B., Gross, R., N'Guessan, P. D., Hocke, A. C., Hammerschmidt, S., Mitchell, T. J., Rosseau, S., Suttorp, N., & Hippenstiel, S. 2004, "Streptococcus pneumoniae-Induced Caspase 6-Dependent Apoptosis in Lung Epithelium", *Infect.Immun.*, vol. 72, no. 9, pp. 4940-4947.

Schmittgen, T. D. 2001, "Real-time quantitative PCR", *Methods*, vol. 25, no. 4, pp. 383-385.

Schultz, M. J., Rijneveld, A. W., Florquin, S., Edwards, C. K., Dinarello, C. A., & van der, P. T. 2002, "Role of interleukin-1 in the pulmonary immune response during Pseudomonas aeruginosa pneumonia", *Am.J.Physiol Lung Cell Mol.Physiol*, vol. 282, no. 2, p. L285-L290.

Schulz, C., Kratzel, K., Wolf, K., Schroll, S., Kohler, M., & Pfeifer, M. 2004, "Activation of bronchial epithelial cells in smokers without airway obstruction and patients with COPD", *Chest*, vol. 125, no. 5, pp. 1706-1713.

Scott, D. T., Lam, F. Y., & Ferrell, W. R. 1994, "Acute joint inflammation--mechanisms and mediators", *Gen.Pharmacol.*, vol. 25, no. 7, pp. 1285-1296.

Sewnath, M. E., Olszyna, D. P., Birjmohun, R., ten Kate, F. J., Gouma, D. J., & van der, P. T. 2001, "IL-10-deficient mice demonstrate multiple organ failure and increased mortality during Escherichia coli peritonitis despite an accelerated bacterial clearance", *J.Immunol.*, vol. 166, no. 10, pp. 6323-6331.

Shankar-Sinha, S., Valencia, G. A., Janes, B. K., Rosenberg, J. K., Whitfield, C., Bender, R. A., Standiford, T. J., & Younger, J. G. 2004, "The Klebsiella pneumoniae O antigen contributes to bacteremia and lethality during murine pneumonia", *Infect.Immun.*, vol. 72, no. 3, pp. 1423-1430.

Shanley, T. P., Vasi, N., & Denenberg, A. 2000, "Regulation of chemokine expression by IL-10 in lung inflammation", *Cytokine*, vol. 12, no. 7, pp. 1054-1064.

Shanley, T. P., Warner, R. L., & Ward, P. A. 1995, "The role of cytokines and adhesion molecules in the development of inflammatory injury", *Mol.Med.Today*, vol. 1, no. 1, pp. 40-45.

Shaver, J. R., O'Connor, J. J., Pollice, M., Cho, S. K., Kane, G. C., Fish, J. E., & Peters, S. P. 1995, "Pulmonary inflammation after segmental ragweed challenge in allergic asthmatic and nonasthmatic subjects", *Am.J.Respir.Crit Care Med.*, vol. 152, no. 4 Pt 1, pp. 1189-1197.

Shimabukuro, D. W., Sawa, T., & Gropper, M. A. 2003, "Injury and repair in lung and airways", *Crit Care Med.*, vol. 31, no. 8 Suppl, p. S524-S531.

Shimamoto, Y., Kitamura, H., Hoshi, H., Kazusaka, A., Funae, Y., Imaoka, S., Saito, M., & Fujita, S. 1998, "Differential alterations in levels of hepatic microsomal

cytochrome P450 isozymes following intracerebroventricular injection of bacterial lipopolysaccharide in rats", *Arch.Toxicol.*, vol. 72, no. 8, pp. 492-498.

Shultz, M. A., Zhang, L., Gu, Y. Z., Baker, G. L., Fannuchi, M. V., Padua, A. M., Gurske, W. A., Morin, D., Penn, S. G., Jovanovich, S. B., Plopper, C. G., & Buckpitt, A. R. 2004, "Gene expression analysis in response to lung toxicants: I. Sequencing and microarray development", *Am.J.Respir.Cell Mol.Biol.*, vol. 30, no. 3, pp. 296-310.

Sielaff, T. D., Sugerman, H. J., Tatum, J. L., Kellum, J. M., & Blocher, C. R. 1987, "Treatment of porcine Pseudomonas ARDS with combination drug therapy", *J.Trauma*, vol. 27, no. 12, pp. 1313-1322.

Skrypina, N. A., Timofeeva, A. V., Khaspekov, G. L., Savochkina, L. P., & Beabealashvilli, R. S. 2003, "Total RNA suitable for molecular biology analysis", *J.Biotechnol.*, vol. 105, no. 1-2, pp. 1-9.

Soria, J. C., Rodriguez, M., Liu, D. D., Lee, J. J., Hong, W. K., & Mao, L. 2002, "Aberrant promoter methylation of multiple genes in bronchial brush samples from former cigarette smokers", *Cancer Res.*, vol. 62, no. 2, pp. 351-355.

Southern, E., Mir, K., & Shchepinov, M. 1999, "Molecular interactions on microarrays", *Nat.Genet.*, vol. 21, no. 1 Suppl, pp. 5-9.

Southern, E. M. 1975, "Detection of specific sequences among DNA fragments separated by gel electrophoresis", *J.Mol.Biol.*, vol. 98, no. 3, pp. 503-517.

Standiford, T. J., Kunkel, S. L., Basha, M. A., Chensue, S. W., Lynch, J. P., III, Toews, G. B., Westwick, J., & Strieter, R. M. 1990, "Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung", *J.Clin.Invest*, vol. 86, no. 6, pp. 1945-1953.

Standiford, T. J., Strieter, R. M., Greenberger, M. J., & Kunkel, S. L. 1996, "Expression and regulation of chemokines in acute bacterial pneumonia", *Biol.Signals*, vol. 5, no. 4, pp. 203-208.

Standiford, T. J., Strieter, R. M., Lukacs, N. W., & Kunkel, S. L. 1995, "Neutralization of IL-10 increases lethality in endotoxemia. Cooperative effects of macrophage inflammatory protein-2 and tumor necrosis factor", *J.Immunol.*, vol. 155, no. 4, pp. 2222-2229.

Starnes, H. F., Jr., Pearce, M. K., Tewari, A., Yim, J. H., Zou, J. C., & Abrams, J. S. 1990, "Anti-IL-6 monoclonal antibodies protect against lethal Escherichia coli

infection and lethal tumor necrosis factor-alpha challenge in mice", *J.Immunol.*, vol. 145, no. 12, pp. 4185-4191.

Steinhauser, M. L., Hogaboam, C. M., Kunkel, S. L., Lukacs, N. W., Strieter, R. M., & Standiford, T. J. 1999, "IL-10 is a major mediator of sepsis-induced impairment in lung antibacterial host defense", *J.Immunol.*, vol. 162, no. 1, pp. 392-399.

Strieter, R. M., Chensue, S. W., Basha, M. A., Standiford, T. J., Lynch, J. P., Baggiolini, M., & Kunkel, S. L. 1990, "Human alveolar macrophage gene expression of interleukin-8 by tumor necrosis factor-alpha, lipopolysaccharide, and interleukin-1 beta", *Am.J.Respir.Cell Mol.Biol.*, vol. 2, no. 4, pp. 321-326.

Strieter, R. M., Kunkel, S. L., & Bone, R. C. 1993, "Role of tumor necrosis factor-alpha in disease states and inflammation", *Crit Care Med.*, vol. 21, no. 10 Suppl, p. S447-S463.

Sun, Y., Zhang, J. Q., Zhang, J., & Ramires, F. J. 1998, "Angiotensin II, transforming growth factor-beta1 and repair in the infarcted heart", *J.Mol.Cell Cardiol.*, vol. 30, no. 8, pp. 1559-1569.

Sur, S., Kita, H., Gleich, G. J., Chenier, T. C., & Hunt, L. W. 1996, "Eosinophil recruitment is associated with IL-5, but not with RANTES, twenty-four hours after allergen challenge", *J.Allergy Clin.Immunol.*, vol. 97, no. 6, pp. 1272-1278.

Susskind, H., Chanana, A. D., Joel, D. D., Janoff, A., & Brill, A. B. 1985, "Assessment of elastase-induced structural and functional changes in sheep lungs", *Bull.Eur.Physiopathol.Respir.*, vol. 21, no. 2, pp. 149-157.

Swinney, D. C., Xu, Y. Z., Scarafia, L. E., Lee, I., Mak, A. Y., Gan, Q. F., Ramesha, C. S., Mulkins, M. A., Dunn, J., So, O. Y., Biegel, T., Dinh, M., Volkel, P., Barnett, J., Dalrymple, S. A., Lee, S., & Huber, M. 2002, "A small molecule ubiquitination inhibitor blocks NF-kappa B-dependent cytokine expression in cells and rats", *J.Biol.Chem.*, vol. 277, no. 26, pp. 23573-23581.

Taha, R., Hamid, Q., Cameron, L., & Olivenstein, R. 2003, "T helper type 2 cytokine receptors and associated transcription factors GATA-3, c-MAF, and signal transducer and activator of transcription factor-6 in induced sputum of atopic asthmatic patients", *Chest*, vol. 123, no. 6, pp. 2074-2082.

Takahashi, F., Takahashi, K., Okazaki, T., Maeda, K., Ienaga, H., Maeda, M., Kon, S., Uede, T., & Fukuchi, Y. 2001, "Role of osteopontin in the pathogenesis of bleomycin-induced pulmonary fibrosis", *Am.J.Respir.Cell Mol.Biol.*, vol. 24, no. 3, pp. 264-271.

Takashima, K., Tateda, K., Matsumoto, T., Iizawa, Y., Nakao, M., & Yamaguchi, K. 1997, "Role of tumor necrosis factor alpha in pathogenesis of pneumococcal pneumonia in mice", *Infect.Immun.*, vol. 65, no. 1, pp. 257-260.

Takizawa, H. 1998, "Airway epithelial cells as regulators of airway inflammation (Review)", *Int.J.Mol.Med.*, vol. 1, no. 2, pp. 367-378.

Takizawa, H., Abe, S., Ohtoshi, T., Kawasaki, S., Takami, K., Desaki, M., Sugawara, I., Hashimoto, S., Azuma, A., Nakahara, K., & Kudoh, S. 2000, "Diesel exhaust particles up-regulate expression of intercellular adhesion molecule-1 (ICAM-1) in human bronchial epithelial cells", *Clin.Exp.Immunol.*, vol. 120, no. 2, pp. 356-362.

Takizawa, H., Tanaka, M., Takami, K., Ohtoshi, T., Ito, K., Satoh, M., Okada, Y., Yamasawa, F., Nakahara, K., & Umeda, A. 2001, "Increased expression of transforming growth factor-beta1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD)", *Am.J.Respir.Crit Care Med.*, vol. 163, no. 6, pp. 1476-1483.

Tamura, G. S., Kuypers, J. M., Smith, S., Raff, H., & Rubens, C. E. 1994, "Adherence of group B streptococci to cultured epithelial cells: roles of environmental factors and bacterial surface components", *Infect.Immun.*, vol. 62, no. 6, pp. 2450-2458.

Terashima, T., Matsubara, H., Nakamura, M., Sakamaki, F., Waki, Y., Soejima, K., Tasaka, S., Nakamura, H., Sayama, K., Ishizaka, A., & Kanazawa, M. 1996, "Local Pseudomonas instillation induces contralateral lung injury and plasma cytokines", *Am.J.Respir.Crit Care Med.*, vol. 153, no. 5, pp. 1600-1605.

Thomas, A., Sachse, K., Farnir, F., Dizier, I., Mainil, J., & Linden, A. 2003, "Adherence of Mycoplasma bovis to bovine bronchial epithelial cells", *Microb.Pathog.*, vol. 34, no. 3, pp. 141-148.

Thomas, P. S. 2001, "Tumour necrosis factor-alpha: the role of this multifunctional cytokine in asthma", *Immunol.Cell Biol.*, vol. 79, no. 2, pp. 132-140.

Thomassen, M. J., Divis, L. T., & Fisher, C. J. 1996, "Regulation of human alveolar macrophage inflammatory cytokine production by interleukin-10", *Clin.Immunol.Immunopathol.*, vol. 80, no. 3 Pt 1, pp. 321-324.

Tindberg, N., Baldwin, H. A., Cross, A. J., & Ingelman-Sundberg, M. 1996, "Induction of cytochrome P450 2E1 expression in rat and gerbil astrocytes by inflammatory factors and ischemic injury", *Mol.Pharmacol.*, vol. 50, no. 5, pp. 1065-1072.

Toews, G. B. 2001, "Cytokines and the lung", *Eur.Respir.J.Suppl*, vol. 34, pp. 3s-17s.

Tomkins, A. 2003, "Assessing micronutrient status in the presence of inflammation", *J.Nutr.*, vol. 133, no. 5 Suppl 2, pp. 1649S-1655S.

Traber, D. L., Lentz, C. W., Traber, L. D., & Herndon, D. N. 1992, "Lymph and blood flow responses in central airways", *Am.Rev.Respir.Dis.*, vol. 146, no. 5 Pt 2, p. S15-S18.

Tran, K. T., Griffith, L., & Wells, A. 2004, "Extracellular matrix signaling through growth factor receptors during wound healing", *Wound.Repair Regen.*, vol. 12, no. 3, pp. 262-268.

Trapnell, B. C. 1993, "Quantitative evaluation of gene expression in freshly isolated human respiratory epithelial cells", *Am.J.Physiol*, vol. 264, no. 3 Pt 1, p. L199-L212.

Truettner, J., Schmidt-Kastner, R., Busto, R., Alonso, O. F., Loor, J. Y., Dietrich, W. D., & Ginsberg, M. D. 1999, "Expression of brain-derived neurotrophic factor, nerve growth factor, and heat shock protein HSP70 following fluid percussion brain injury in rats", *J.Neurotrauma*, vol. 16, no. 6, pp. 471-486.

Tsai, C. A., Hsueh, H. M., & Chen, J. J. 2003, "Estimation of false discovery rates in multiple testing: application to gene microarray data", *Biometrics*, vol. 59, no. 4, pp. 1071-1081.

Tseng, W. Y., Tsao, C. F., Ko, C. C., & Huang, H. T. 2001, "Local capsaicin application to the stellate ganglion and stellatectomy attenuate neurogenic inflammation in rat bronchi", *Auton.Neurosci.*, vol. 94, no. 1-2, pp. 25-33.

Tsuruoka, M., Arai, Y. C., Nomura, H., Matsutani, K., & Willis, W. D. 2003, "Unilateral hindpaw inflammation induces bilateral activation of the locus coeruleus and the nucleus subcoeruleus in the rat", *Brain Res.Bull.*, vol. 61, no. 2, pp. 117-123.

Ulich, T. R., Watson, L. R., Yin, S. M., Guo, K. Z., Wang, P., Thang, H., & del Castillo, J. 1991, "The intratracheal administration of endotoxin and cytokines. I. Characterization of LPS-induced IL-1 and TNF mRNA expression and the LPS-, IL-1-, and TNF-induced inflammatory infiltrate", *Am.J.Pathol.*, vol. 138, no. 6, pp. 1485-1496.

van der, P. T., Keogh, C. V., Buurman, W. A., & Lowry, S. F. 1997, "Passive immunization against tumor necrosis factor-alpha impairs host defense during pneumococcal pneumonia in mice", *Am.J.Respir.Crit Care Med.*, vol. 155, no. 2, pp. 603-608.

van der, P. T., Marchant, A., Buurman, W. A., Berman, L., Keogh, C. V., Lazarus, D. D., Nguyen, L., Goldman, M., Moldawer, L. L., & Lowry, S. F. 1995, "Endogenous IL-10 protects mice from death during septic peritonitis", *J.Immunol.*, vol. 155, no. 11, pp. 5397-5401.

van der, P. T., Marchant, A., Keogh, C. V., Goldman, M., & Lowry, S. F. 1996, "Interleukin-10 impairs host defense in murine pneumococcal pneumonia", *J.Infect.Dis.*, vol. 174, no. 5, pp. 994-1000.

Vandesompele, J., De Paepe, A., & Speleman, F. 2002, "Elimination of primer-dimer artifacts and genomic coamplification using a two-step SYBR green I real-time RT-PCR", *Anal.Biochem.*, vol. 303, no. 1, pp. 95-98.

Vermehren, C., Jorgensen, K., Schiffelers, R., & Frokjaer, S. 2001, "Activity of mammalian secreted phospholipase A(2) from inflammatory peritoneal fluid towards PEG-liposomes. Early indications", *Int.J.Pharm.*, vol. 214, no. 1-2, pp. 93-98.

Vernooy, J. H., Dentener, M. A., van Suylen, R. J., Buurman, W. A., & Wouters, E. F. 2001, "Intratracheal instillation of lipopolysaccharide in mice induces apoptosis in bronchial epithelial cells: no role for tumor necrosis factor-alpha and infiltrating neutrophils", *Am.J.Respir.Cell Mol.Biol.*, vol. 24, no. 5, pp. 569-576.

Vicente-Manzanares, M., Sancho, D., Yanez-Mo, M., & Sanchez-Madrid, F. 2002, "The leukocyte cytoskeleton in cell migration and immune interactions", *Int.Rev.Cytol.*, vol. 216, pp. 233-289.

Villani, G., Greco, M., Papa, S., & Attardi, G. 1998, "Low reserve of cytochrome c oxidase capacity in vivo in the respiratory chain of a variety of human cell types", *J.Biol.Chem.*, vol. 273, no. 48, pp. 31829-31836.

Wagner, E. M. & Mitzner, W. A. 1990, "Bronchial circulatory reversal of methacholine-induced airway constriction", *J.Appl.Physiol*, vol. 69, no. 4, pp. 1220-1224.

Walters, D. M., Breysse, P. N., Schofield, B., & Wills-Karp, M. 2002, "Complement factor 3 mediates particulate matter-induced airway hyperresponsiveness", *Am.J.Respir.Cell Mol.Biol.*, vol. 27, no. 4, pp. 413-418.

Weissman, D. N., Bice, D. E., Muggenburg, B. A., Haley, P. J., Shopp, G. M., & Schuyler, M. R. 1992, "Primary immunization in the canine lung. Soluble antigen induces a localized response", *Am.Rev.Respir.Dis.*, vol. 145, no. 1, pp. 6-12.

Werlen, C., Py, P., & Haab, P. 1984, "Alveolar-arterial equilibration in the lung of sheep", *Respir.Physiol*, vol. 55, no. 2, pp. 205-221.

Wesselius, L. J., Nelson, M. E., Bailey, K., & O'Brien-Ladner, A. R. 1997, "Rapid lung cytokine accumulation and neutrophil recruitment after lipopolysaccharide inhalation by cigarette smokers and nonsmokers", *J.Lab Clin.Med.*, vol. 129, no. 1, pp. 106-114.

West, M. A. & Heagy, W. 2002, "Endotoxin tolerance: A review", *Crit Care Med.*, vol. 30, no. 1 Supp, p. S64-S73.

Wheeler, A. P., Hardie, W. D., & Bernard, G. 1990, "Studies of an antiendotoxin antibody in preventing the physiologic changes of endotoxemia in awake sheep", *Am.Rev.Respir.Dis.*, vol. 142, no. 4, pp. 775-781.

Wheeler, A. P., Hardie, W. D., & Bernard, G. R. 1992, "The role of cyclooxygenase products in lung injury induced by tumor necrosis factor in sheep", *Am.Rev.Respir.Dis.*, vol. 145, no. 3, pp. 632-639.

White, J. C., Nelson, S., Winkelstein, J. A., Booth, F. V., & Jakab, G. J. 1986, "Impairment of antibacterial defense mechanisms of the lung by extrapulmonary infection", *J.Infect.Dis.*, vol. 153, no. 2, pp. 202-208.

Wiethoff, A. J., Reed, K. L., Webb, T. R., & Warheit, D. B. 2003, "Assessing the role of neutrophil apoptosis in the resolution of particle-induced pulmonary inflammation", *Inhal.Toxicol.*, vol. 15, no. 12, pp. 1231-1246.

Wigle, D. A., Rossant, J., & Jurisica, I. 2001, "Mining mouse microarray data", *Genome Biol.*, vol. 2, no. 7, p. REVIEWS1019.

Wolfgang, M. C., Jyot, J., Goodman, A. L., Ramphal, R., & Lory, S. 2004, "Pseudomonas aeruginosa regulates flagellin expression as part of a global response to airway fluid from cystic fibrosis patients", *Proc.Natl.Acad.Sci.U.S.A*, vol. 101, no. 17, pp. 6664-6668.

Woodall, C. J., Maclaren, L. J., & Watt, N. J. 1997, "Differential levels of mRNAs for cytokines, the interleukin-2 receptor and class II DR/DQ genes in ovine interstitial pneumonia induced by maedi visna virus infection", *Vet.Pathol.*, vol. 34, no. 3, pp. 204-211.

Wool, I. G. 1996, "Extraribosomal functions of ribosomal proteins", *Trends Biochem.Sci.*, vol. 21, no. 5, pp. 164-165.

Wool, I. G., Chan, Y. L., & Gluck, A. 1995, "Structure and evolution of mammalian ribosomal proteins", *Biochem.Cell Biol.*, vol. 73, no. 11-12, pp. 933-947.

Worthen, G. S., Haslett, C., Rees, A. J., Gumbay, R. S., Henson, J. E., & Henson, P. M. 1987, "Neutrophil-mediated pulmonary vascular injury. Synergistic effect of trace amounts of lipopolysaccharide and neutrophil stimuli on vascular permeability and neutrophil sequestration in the lung", *Am.Rev.Respir.Dis.*, vol. 136, no. 1, pp. 19-28.

Wright, J. R., Borron, P., Brinker, K. G., & Folz, R. J. 2001, "Surfactant Protein A: regulation of innate and adaptive immune responses in lung inflammation", *Am.J.Respir.Cell Mol.Biol.*, vol. 24, no. 5, pp. 513-517.

Wright, S. D., Detmers, P. A., Aida, Y., Adamowski, R., Anderson, D. C., Chad, Z., Kabbash, L. G., & Pabst, M. J. 1990a, "CD18-deficient cells respond to lipopolysaccharide in vitro", *J.Immunol.*, vol. 144, no. 7, pp. 2566-2571.

Wright, S. D., Ramos, R. A., Hermanowski-Vosatka, A., Rockwell, P., & Detmers, P. A. 1991, "Activation of the adhesive capacity of CR3 on neutrophils by endotoxin: dependence on lipopolysaccharide binding protein and CD14", *J.Exp.Med.*, vol. 173, no. 5, pp. 1281-1286.

Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., & Mathison, J. C. 1990b, "CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein", *Science*, vol. 249, no. 4975, pp. 1431-1433.

Wu, X., Herndon, D. N., & Wolf, S. E. 2003, "Growth hormone down-regulation of Interleukin-1beta and Interleukin-6 induced acute phase protein gene expression is associated with increased gene expression of suppressor of cytokine signal-3", *Shock*, vol. 19, no. 4, pp. 314-320.

Xia, Z., Herijgers, P., Nishida, T., Ozaki, S., Wouters, P., & Flameng, W. 2003, "Remote preconditioning lessens the deterioration of pulmonary function after repeated coronary artery occlusion and reperfusion in sheep: Un preconditionnement eloigne diminue la deterioration de la fonction pulmonaire apres l'occlusion et la reperfusion repetees de l'artere coronaire", *Can.J.Anaesth.*, vol. 50, no. 5, pp. 481-488.

Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X. F., & Achong, M. K. 1998, "IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses", *J.Clin.Invest*, vol. 101, no. 2, pp. 311-320.

Xing, Z., Jordana, M., Gauldie, J., & Wang, J. 1999, "Cytokines and pulmonary inflammatory and immune diseases", *Histol.Histopathol.*, vol. 14, no. 1, pp. 185-201.

Xing, Z., Jordana, M., Kirpalani, H., Driscoll, K. E., Schall, T. J., & Gauldie, J. 1994, "Cytokine expression by neutrophils and macrophages in vivo: endotoxin induces tumor necrosis factor-alpha, macrophage inflammatory protein-2, interleukin-1 beta, and interleukin-6 but not RANTES or transforming growth factor-beta 1 mRNA expression in acute lung inflammation", *Am.J.Respir.Cell Mol.Biol.*, vol. 10, no. 2, pp. 148-153.

Xing, Z., Kirpalani, H., Torry, D., Jordana, M., & Gauldie, J. 1993, "Polymorphonuclear leukocytes as a significant source of tumor necrosis factor-alpha in endotoxin-challenged lung tissue", *Am.J.Pathol.*, vol. 143, no. 4, pp. 1009-1015.

Yamada, H., Miyazaki, H., Kikuchi, T., Fujimoto, J., & Kudoh, I. 2000, "Acid instillation enhances the inflammatory response to subsequent lipopolysaccharide challenge in rats", *Am.J.Respir.Crit Care Med.*, vol. 162, no. 4 Pt 1, pp. 1366-1371.

Yamamoto, H., Sedgwick, J. B., & Busse, W. W. 1998, "Differential regulation of eosinophil adhesion and transmigration by pulmonary microvascular endothelial cells", *J.Immunol.*, vol. 161, no. 2, pp. 971-977.

Yamamoto, T., Kajikawa, O., Martin, T. R., Sharar, S. R., Harlan, J. M., & Winn, R. K. 1998, "The role of leukocyte emigration and IL-8 on the development of lipopolysaccharide-induced lung injury in rabbits", *J.Immunol.*, vol. 161, no. 10, pp. 5704-5709.

Yamanaka, Y., Tamari, M., Nakahata, T., & Nakamura, Y. 2001, "Gene expression profiles of human small airway epithelial cells treated with low doses of 14- and 16-membered macrolides", *Biochem.Biophys.Res.Commun.*, vol. 287, no. 1, pp. 198-203.

Yang, G. P., Ross, D. T., Kuang, W. W., Brown, P. O., & Weigel, R. J. 1999, "Combining SSH and cDNA microarrays for rapid identification of differentially expressed genes", *Nucleic Acids Res.*, vol. 27, no. 6, pp. 1517-1523.

Yang, M., Hogan, S. P., Henry, P. J., Matthaei, K. I., McKenzie, A. N., Young, I. G., Rothenberg, M. E., & Foster, P. S. 2001, "Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor-alpha chain and STAT-6 independently of IL-5 and eotaxin", *Am.J.Respir.Cell Mol.Biol.*, vol. 25, no. 4, pp. 522-530.

Yang, M. C., Yang, J. J., McIndoe, R. A., & She, J. X. 2003, "Microarray experimental design: power and sample size considerations", *Physiol Genomics*, vol. 16, no. 1, pp. 24-28.

Yates, A., Bergmann, C., Van Hemmen, J. L., Stark, J., & Callard, R. 2000, "Cytokine-modulated regulation of helper T cell populations", *J.Theor.Biol.*, vol. 206, no. 4, pp. 539-560.

Yoshikawa, H., Yasu, T., Ueki, H., Oyamada, T., Oishi, H., Anzai, T., Oikawa, M., & Yoshikawa, T. 2003, "Pneumonia in horses induced by intrapulmonary inoculation of Streptococcus equi subsp. zooepidemicus", *J.Vet.Med.Sci.*, vol. 65, no. 7, pp. 787-792.

Yoshiyama, M., Takeuchi, K., Hanatani, A., Kim, S., Omura, T., Toda, I., Teragaki, M., Akioka, K., Iwao, H., & Yoshikawa, J. 1997, "Differences in expression of sarcoplasmic reticulum Ca2+-ATPase and Na+-Ca2+ exchanger genes between adjacent and remote noninfarcted myocardium after myocardial infarction", *J.Mol.Cell Cardiol.*, vol. 29, no. 1, pp. 255-264.

Younger, J. G., Shankar-Sinha, S., Mickiewicz, M., Brinkman, A. S., Valencia, G. A., Sarma, J. V., Younkin, E. M., Standiford, T. J., Zetoune, F. S., & Ward, P. A. 2003, "Murine complement interactions with Pseudomonas aeruginosa and their consequences during pneumonia", *Am.J.Respir.Cell Mol.Biol.*, vol. 29, no. 4, pp. 432-438.

Zangrilli, J. G., Shaver, J. R., Cirelli, R. A., Cho, S. K., Garlisi, C. G., Falcone, A., Cuss, F. M., Fish, J. E., & Peters, S. P. 1995, "sVCAM-1 levels after segmental antigen challenge correlate with eosinophil influx, IL-4 and IL-5 production, and the late phase response", *Am.J.Respir.Crit Care Med.*, vol. 151, no. 5, pp. 1346-1353.

Zeldin, D. C., Wohlford-Lenane, C., Chulada, P., Bradbury, J. A., Scarborough, P. E., Roggli, V., Langenbach, R., & Schwartz, D. A. 2001, "Airway inflammation and responsiveness in prostaglandin H synthase-deficient mice exposed to bacterial lipopolysaccharide", *Am.J.Respir.Cell Mol.Biol.*, vol. 25, no. 4, pp. 457-465.

Zhai, Q. H., Futrell, N., & Chen, F. J. 1997, "Gene expression of IL-10 in relationship to TNF-alpha, IL-1beta and IL-2 in the rat brain following middle cerebral artery occlusion", *J.Neurol.Sci.*, vol. 152, no. 2, pp. 119-124.

Zhang, B., Nie, X., Xiao, B., Xiang, J., Shen, S., Gong, J., Zhou, M., Zhu, S., Zhou, J., Qian, J., Lu, H., He, X., Li, X., Hu, G., & Li, G. 2003, "Identification of tissue-specific genes in nasopharyngeal epithelial tissue and differentially expressed genes in nasopharyngeal carcinoma by suppression subtractive hybridization and cDNA microarray", *Genes Chromosomes.Cancer*, vol. 38, no. 1, pp. 80-90.

Zhao, B., Bowden, R. A., Stavchansky, S. A., & Bowman, P. D. 2001, "Human endothelial cell response to gram-negative lipopolysaccharide assessed with cDNA microarrays", *Am.J.Physiol Cell Physiol*, vol. 281, no. 5, p. C1587-C1595.

Zhao, B., Stavchansky, S. A., Bowden, R. A., & Bowman, P. D. 2003, "Effect of interleukin-1beta and tumor necrosis factor-alpha on gene expression in human endothelial cells", *Am.J.Physiol Cell Physiol*, vol. 284, no. 6, p. C1577-C1583.

Zimmermann, N., King, N. E., Laporte, J., Yang, M., Mishra, A., Pope, S. M., Muntel, E. E., Witte, D. P., Pegg, A. A., Foster, P. S., Hamid, Q., & Rothenberg, M. E. 2003, "Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis", *J.Clin.Invest*, vol. 111, no. 12, pp. 1863-1874.

Zisman, D. A., Kunkel, S. L., Strieter, R. M., Gauldie, J., Tsai, W. C., Bramson, J., Wilkowski, J. M., Bucknell, K. A., & Standiford, T. J. 1997, "Anti-interleukin-12 therapy protects mice in lethal endotoxemia but impairs bacterial clearance in murine Escherichia coli peritoneal sepsis", *Shock*, vol. 8, no. 5, pp. 349-356.

Zou, J., Young, S., Zhu, F., Gheyas, F., Skeans, S., Wan, Y., Wang, L., Ding, W., Billah, M., McClanahan, T., Coffman, R. L., Egan, R., & Umland, S. 2002, "Microarray profile of differentially expressed genes in a monkey model of allergic asthma", *Genome Biol.*, vol. 3, no. 5, p. research0020.

Appendices

This section contains appendices I and V in paper format. Appendices II-IV are included on the CD-ROM disc in the back cover. Instructions for accessing each file type, and relevant programs to be used appear within each directory as a Microsoft Word file. The file and directory structure used on the CD is as follows:

-Appendix II

-Brushing movie clip

This directory contains a movie file recorded via a bronchoscope showing the brushing action used to obtain epithelial cells.

-RNA data

This directory contains original files from the Agilent Bioanalyzer software detailing the analysis of total RNA taken from the epithelial brushings. It also contains image files of the fluorescence traces and 'false gels' from those files.

-qPCR data

This directory contains the original Opticon Monitor files with real-time PCR data on each experiment. It also contains a spreadsheet with all original data derived from those files and used in analysis.

-Appendix III

-Homology search results

This directory contains a spreadsheet file detailing the ovine-bovine library homolog list and relevant data for the bovine library clones selected for inclusion on the microarray.

-Microarray PCR data

This directory contains a spreadsheet file with all of the quality control data for the PCR products used to print the microarray

-Microarray structure details

This directory contains the layout file for the microarray used by the data acquisition software to identify and label the array elements.

-aRNA data

This directory contains original files from the Agilent Bioanalyzer software detailing the analysis of amplified RNA taken from the epithelial brushings. It also contains image files of the fluorescence traces and 'false gels' from those files.

-Microarray images

This directory contains all of the scanned microarray images used for data acquisition.

-Appendix IV

-Raw array data

-Direct / Remote segment microarray data

These directories contain spreadsheet files with all of the raw data acquired from the microarray images and used in the data analysis process.

Appendix I:

Table 1: Absolute cell counts ($x10^6$ cells / ml blood)in blood samples taken from sheep at baseline (0 hours) and 6 hours following LPS challenge (6 hours).

Sheep	Total PMN (0 h)	Total PMN (6 h)	Total lymphocytes (0 h)	Total lymphocytes (6 h)	Total monocytes (0 h)	Total monocytes (6 h)
451	3.44	8.05	4.90	3.66	0.09	0.49
0631P	0	3.83	4.70	1.46	0.90	0.11
0681P	2.11	5.81	6.72	2.55	0.77	0.44
0687P	2.42	5.26	3.80	1.8	0.62	0.07
0693P	0.78	6.84	9.55	4.32	0.78	0.72
0757P	0.05	0.6	3.96	3.75	0.54	0.6
0861P	0	3.8	11.13	1.15	1.38	0.05
S12	0.63	0.63	3.56	5.48	0.09	0.19
S41	2.73	3.71	2.26	2.54	0.29	0.20
S46	2.02	2.30	1.59	1.48	0.26	0.33
S9	0.13	2.60	6.16	1.57	0.20	0.20
X26	0.99	3.18	5.15	2.34	0.20	0.48
Median	0.88	3.75	4.80	2.44	0.42	0.26

Table 2: Rectal temperatures (°C) of sheep taken at baseline (0 hours) and at 6 hours post-LPS challenge (6 hours).

Sheep	0 hours	6 hours	Change
451	38.89	39.56	0.67
0631P	38.89	39	0.11
0681P	38.61	39.22	0.61
0687P	39.89	40	0.11
0693P	39.44	39.89	0.45
0757P	39.17	39.44	0.27
0861P	39.11	40.11	1
S12	39	39.89	0.89
S41	38.89	40.44	1.55
S46	39	40.78	1.78
S9	39.11	40.33	1.22
X26	39	40	1
Median	39	39.95	0.78

Appendix IV : Second LPS challenge contra laterally

Aims and Objectives

To determine if the changes in gene expression evinced in the contra lateral lung 6 hours after local LPS instillation modulate that lung's response to an identical challenge.

Introduction

LPS can elicit pro- or anti-inflammatory reactions in the lung. LPS has been shown to promote the production of cytokines by macrophages as well as the up-regulation of adhesion molecules on endothelial cells which promotes immune cell trafficking. LPS is also known to induce the expression of anti-inflammatory enzymes (e.g. superoxide dismutase (SOD), catalase), heat shock proteins and inducible nitric oxide synthase (iNOS).

The preceding chapters demonstrate that local lung inflammation induced by gram-negative bacterial LPS induces a genotypic response in the contra lateral lung. As many aspects of this remote response are anti-inflammatory in nature, it is hypothesised that the phenotype of an acute inflammation induced by direct challenge against such a background would show evidence of down-regulation. Specifically, it is hypothesised that an LPS challenge delivered to the contra lateral lung 6 hours after the primary challenge will result in an attenuated inflammatory response in that lung relative to that observed following the primary challenge.

This model shares analogies with the clinical progression of pulmonary ARDS (Pelosi et al. 2003). Direct challenge followed by a repeat of the same or similar challenge to the same tissue has been shown to result in attenuated inflammatory effects, including reduced neutrophil numbers (Jean et al. 1998). Dehoux et al showed that in the contra lateral lung, the resident macrophages – a critical inflammatory mediator – are hyporeactive following unilateral pneumonia , whilst Muehlstedt et al demonstrated

that traumatic injury induced alterations in local lung effector cell function that could potentially predispose the lungs of injured patients to infection .

It has been postulated that exposure to LPS can be protective to the host and reduce or ablate the harmful effects of LPS-induced inflammation. This suggestion is supported by the reported existence of LPS-induced preconditioning or tolerance . During preconditioning LPS challenge at low levels renders tissues resistant to subsequent challenge with a higher dose of LPS . The protective effect of LPS preconditioning includes decreased mortality, improved lung function, and inhibition of macrophage activity after secondary LPS challenge.

Other organs have also been studied for pre-conditioning effects, and the heart in particular has been a focus for this research. The theory behind pre-conditioning is that a minor injury to an organ will then protect the injured tissue against subsequent damage. Evidence for this in the heart has been provided by Cohen et al using myocardial stunning in a canine model (Cohen & Downey 1990), in the lower limb by Harkin et al (Harkin et al. 2002), and in the liver by Peralta et al (Peralta et al. 2001). Both of the latter groups used an ischemia-reperfusion model to precondition before damaging the limb with a prolonged period of ischemia and then assessed the level of protection afforded by the pre-conditioning injury.

Indeed, the theory has been expanded to include remote pre-conditioning whereby a distant section of the organ to be protected is subject to minor injury and the whole organ receives protection as a consequence. This process is reviewed by Przyclenk (Przyklenk et al. 2003) and is supported by recent evidence published by Xia et al who demonstrated significant protection granted to the heart and lungs against ischemia / reperfusion injury, by transient occlusion of the iliac artery in sheep (Xia et al. 2003). Although preconditioning with LPS is seen in several tissues, the

mechanism underlying the protection against inflammatory stimuli is unclear. LPS is known to elicit an increased expression of endothelial cell adhesion molecules such as the selectins and it is possible that inhibition of production of adhesion molecules accounts for part of the preconditioning response.

Results

Absolute neutrophil counts 6 hours after initial challenge in the direct segment and 6 hours after second challenge in the 'repeat' segment contra lateral to it were both significantly higher than at baseline in the respective segments (direct: $p = 0.018$, $n = 6$; repeat: $p = 0.018$, $n = 6$). There was no statistically significant difference between the absolute number of neutrophils in the directly challenged segments at 6 hours when compared with the numbers in the contra lateral segment at 12 hours (repeat challenge segment) ($p = 0.402$, $n = 6$). However the median fold change from baseline in the repeat segment was 0.55 times that in the direct segment (150 vs. 270) which represents close to a halving of the median change in magnitude of PMN infiltration. No other cell types showed any significant change. Figure V.1 shows the changes in absolute neutrophil numbers in each segment and figure V.2 shows the same data expressed as mean fold change from baseline in each animal sampled in the direct and repeat segments.

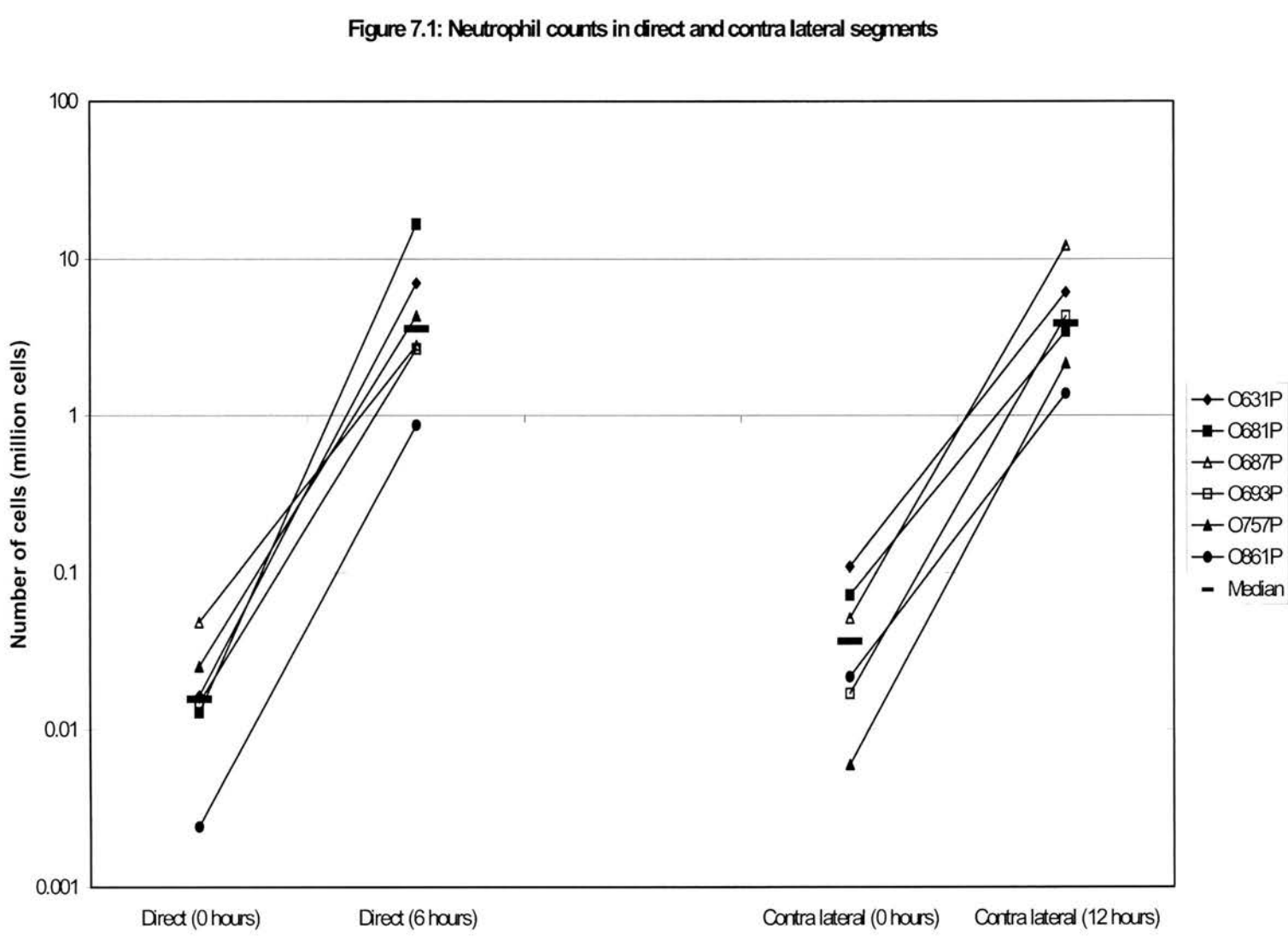


Figure V.1: This figure illustrates, for each animal, the total number of neutrophils present in BALF in the directly challenged and remote segments prior to treatment, and 6 hours post-LPS challenge (x 10^6 cells). Total neutrophil numbers were significantly ($p=0.018$) increased six hours post-LPS challenge in BALF taken from each segment. This represented the major part of the increase in the total cell numbers.

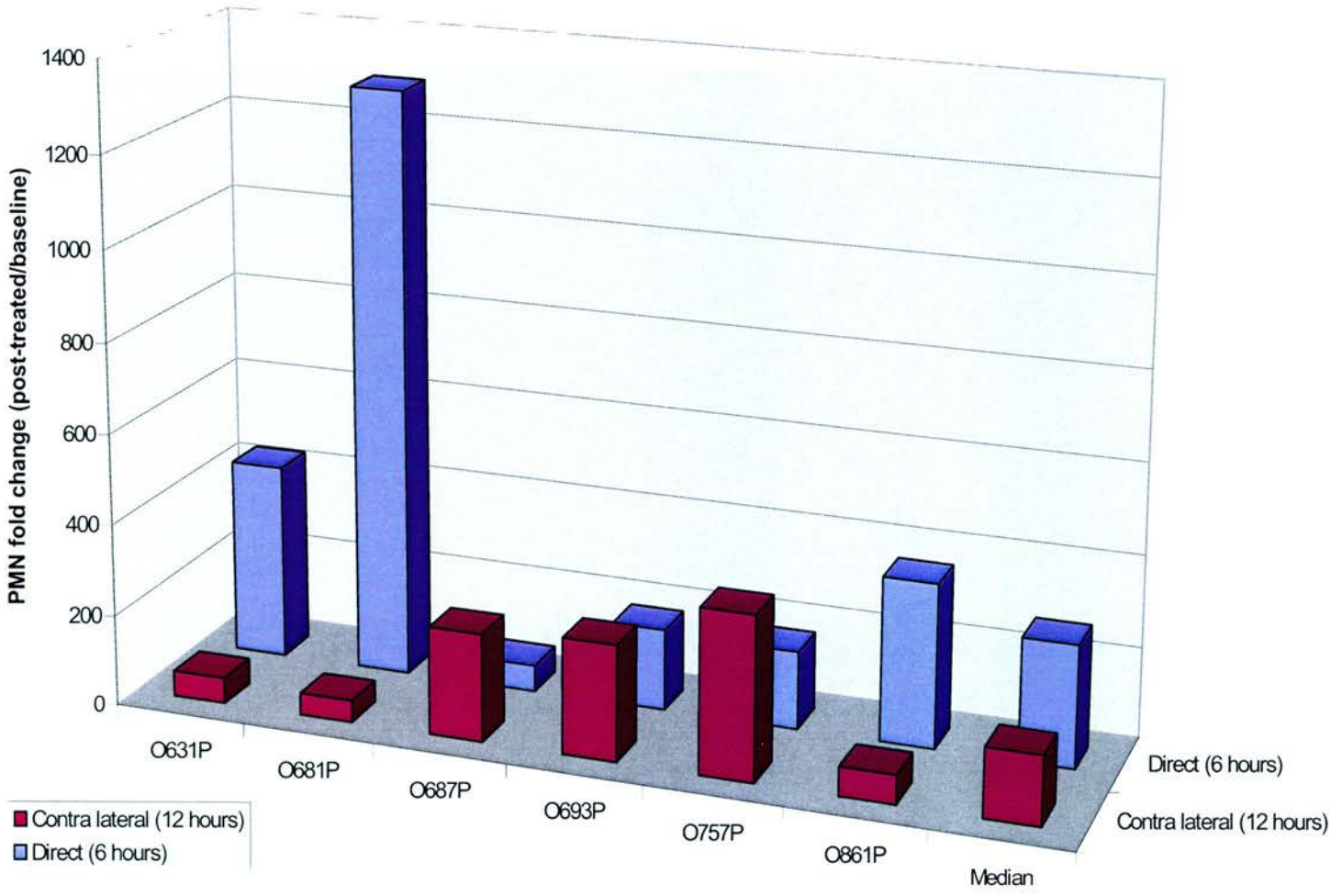


Figure V.2: 3D bar chart displaying, for each animal, the mean fold change in numbers of neutrophils present in BALF after LPS challenge compared with baseline. The median fold change overall is also displayed. Although these data did not reach statistical significance, there is a trend towards down regulation of the neutrophil response in the repeat segment compared with the direct one.

Discussion

The lack of significant evidence for a modulation of the inflammatory response following the challenge in the remote segment does not support the hypothesis stated at the start of this chapter however this result was inconclusive and bears further investigation.

The original intention in this study was to evaluate the comparative genetic changes between the directly challenged lung segment at 6 hours post-LPS challenge and a contra lateral lung segment 6 hours after a second challenge. The previous literature suggested that the initial response elicited by the local LPS challenge would 'precondition' the lung, leading to a protective reduction in the inflammatory response. At the genetic level this response might be expected to include reduced production of pro-inflammatory mediators compared with those seen in the initial challenge. The phenotypic changes in the lung were expected to be somewhat attenuated after preconditioning, but were not expected to be eliminated as this would be contradictory to the previously observed effects of secondary infection in clinical scenarios.

Whilst it is true that PMN numbers in the lung are an accepted and valuable measure of inflammation in the lung, detection of statically significant differences between the two segments would require more samples than were available in this study. An initial power calculation indicated that the number of samples (i.e. sheep) needed to achieve significance would be 17, given a 0.55 fold change in PMN numbers (the level seen in this study) and the variances in sample readings. In order for the sample size used to find significant changes statistically, the PMN numbers in the repeat segment would have to approximately 25% of those in the direct segment, a change that would be

inconsistent with the previously observed models of preconditioning and endotoxin tolerance.

A further factor in the consideration of the results is the severity of the LPS challenge administered. As is clear from the high levels of neutrophils infiltrating the lung space, the challenge is one of considerable potency. With such a potent challenge it is possible that any protection, or indeed vulnerability, induced by the preconditioning of the organ would be rendered undetectable and insufficient in terms of altering the phenotypic outcome of the inflammatory response. Models that have been used to evaluate similar pre-treatment in the lung have utilised different levels of stimulus, with a more potent inflammation as a pre-treatment, followed by relatively mild subsequent challenge.

A future experimental design would benefit from considerations such as an increase in sample size, and some modification of the sampling procedure. A possibility for a future study might be a combined inflammation and infection control study. The evaluation of bacterial clearance in a mortal experiment following an inflammatory pre-treatment would be more closely analogous to the situation in a clinical ARDS progression, and would be of considerable value as a verification in a large animal model of the observation that a pre-treated lung may react to subsequent infection by improving bacterial clearance but increasing risk of mortality. Further consideration should also be given to the length of time between primary and secondary challenges. A longer period of time would be more in keeping with reported observations in clinical settings where mean time between admission and development of nosocomial pneumonia leading to ARDS is approximately 72 hours.

Unfortunately due to time constraint it was not possible to complete the evaluation of the genetic changes in terms of either cytokine expression or microarray analysis,

however the relevant tissue and cellular samples were archived and it is anticipated that this investigation will occur at some point in the future. These investigations may indicate underlying genetic trends whose are not amenable to evaluation on the basis of neutrophil infiltration alone.